

nature

Rewriting history is not possible

Many countries in the West are in confusion about Japan's role in the Second World War, but demands for apologies are generally misplaced; teaching contemporary history is a better course.

THE fiftieth anniversary this week of the ending of the Pacific War, and with it of the Second World War, has prompted a predictable fuss. A recurrent theme has been the demand that "Japan should apologize", largely on behalf of those unfortunate enough to have spent long periods of time as prisoners of war of the Japanese. In Britain, there have been protracted attempts to parse (grammatically and diplomatically) selected phrases in a personal letter from the Japanese prime minister, Mr Tomiichi Murayama, to the British prime minister, Mr John Major. Does "*fukai hansei*" really mean "deep remorse", or merely "profound reflection"? And does the use of the word "*owabi*" in a personal letter mean that the Japanese government has formally apologized or merely that Murayama (well-known as a pacifist) is sorry that there was a war? The fuss would be comic were it not that it is a serious business also.

Times of war are necessarily terrible times. To the extent that the combatants believe they are fighting for their survival, they are able to justify to themselves all kinds of horrible acts. The polite understanding that only the lives of opponents who happen to be wearing military uniforms should be put at risk has been a dead letter since the development of the military bomber, but there were several occasions during the Second World War when targets were chosen for the terror they would engender among civilians. The justification that ordinary people fearful for their lives will persuade their governments to cave in has rarely been sustained. Reprisals by occupying forces against civilian populations they control are also commonplace, but are justified on the grounds that victorious armies have a right to expect loyalty from those in thrall to them. But vindictiveness plays an important part, as recent events in the former Yugoslavia show as vividly as the murder of 15,000 Polish officers in 1943 by Russian forces at Katyn.

Attempts to civilize warfare have not so far been conspicuously successful, although the formal conventions banning the use of inhumane weapons (poisonous gases, for example) may have had some influence during the Second World War. (Combatants may also have calculated that they had as much to lose as to gain from such an extension of their armouries.) The formal convention of the treatment of prisoners has also been modestly successful; the rights of prisoners to be fed and housed safely, to send and receive mail and not to be forced into labour against their will have been widely, if not universally, respected. (Ex-Yugoslavia is again an exception.) There are well-documented complaints

against Japan on such grounds, not assuaged by the consideration that armed forces facing defeat are less able to look after prisoners properly. Thence the demands for apologies.

They are misplaced, but not on the grounds that all combatants behave badly at some stage in the wars in which they are engaged. They do, but so what? The events of the Second World War are indelibly part of our contemporary history. For many of them, reparation is not possible and apology is inappropriate; different people were responsible. What is possible and does matter is that all the participants in wars that are mercifully now ended should separately and openly know about and understand the ways in which their predecessors have exercised responsibility. It must be hoped that Japanese will cultivate the openness about recent history of the Germans in recent decades and now, spectacularly, of the Russians. The history will remain unchanged. □

Nicotine as a drug

Clinton's war on teenage smoking is a just war, but will need imagination as well as toughness.

ROBBED a year ago by the Congress of his favourite cause of health-care reform, President Bill Clinton has declared war on cigarette-smoking by young people in the United States. He has epidemiology and much else on his side. And those who catch the habit young are more likely to suffer the ill-effects. Tobacco-smoking is an abomination, especially among the young, and the administration is right to seek to stamp it out.

There are also obvious difficulties, from the First Amendment (which inhibits control of advertising) to the risks of giving organized crime a slice of the tobacco market (if taxes are put up too high). The lobbies can be relied upon to offer stiff resistance. There are also snags in having the Food and Drug Administration classify nicotine as a drug, unless the administration will go so far as to control marijuana on the same basis, selling restricted supplies of both only to those who register as helpless addicts. But the intrusion into this new market of the state liquor stores enjoying a monopoly on sales of alcohol in many states would only strengthen the reputation of the United States as a kill-joy society. The president's political opponents in the Congress would not like that either. Who can devise an incentive to persuade teenagers not to smoke? □



France and US in late conversion to 'zero-yield' nuclear test ban

Paris. Prospects for the introduction of a Comprehensive Test Ban Treaty (CTBT) were revived last week after France confirmed that it will support a ban on all nuclear tests, no matter how small, from next year (see *Nature* 376, 283; 1995).

The French decision — which has been interpreted as an attempt to turn the tables on critics of its planned series of tests in the South Pacific — was immediately followed by a similar endorsement of a total test ban by the US President Bill Clinton. "I applaud the French statement," he said. "It will make it much easier for us to get a comprehensive test ban."

Indeed, the French and US statements will break the deadlock in negotiations on the CTBT at the Conference on Disarmament in Geneva, where the permanent five nuclear weapons-state members of the United Nations Security Council — France, the United States, China, Russia and the United Kingdom — have been considering introducing a loophole in the ban that would allow low yield nuclear explosions of up to 1 kiloton (see *Nature* 376, 283; 1995).

Although such tests would not allow full testing of conventional nuclear weapons, observers argue that they would provide nuclear states with data that would give them added confidence in a new design. Non-nuclear weapons nations oppose a treaty containing such a loophole, as they consider it would drop disarmament from the goals of the CTBT.

By ruling out large tests, the US and France will make the treaty easier to accept for non-nuclear nations that are either unable or unwilling to carry out such tests. These nations agreed to an indefinite extension of the Nuclear Non-Proliferation Treaty (NPT) earlier this year on the understanding that a test ban would be sufficiently comprehensive to encourage disarmament as well as non-proliferation.

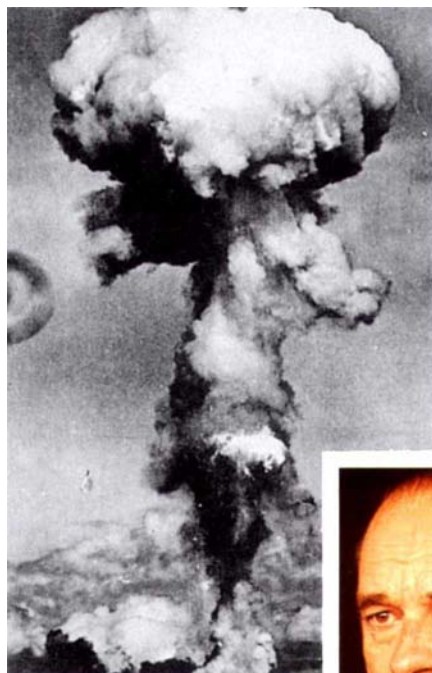
In particular, the clarity of last week's statement by Gérard Errera, the French delegate to the Conference on Disarmament — which said that France would press for a ban on "all tests of nuclear arms and all nuclear explosions" — if confirmed in a formal treaty, would dispel suspicions that France intends to develop a new generation of weapons.

"We are very happy with the statement, it's as good as we hoped but better than we expected," says Patricia Lewis, head of the London-based Verification Technology Information Centre (VERTIC), who adds, however, that "we're still trying to read between the lines".

Gareth Evans, the foreign minister of

Australia — which has pressed for a zero-yield test-ban treaty — said France had taken "an important step forward", adding that if the decision is confirmed in the treaty it would be a "major breakthrough, exactly the commitment that we have asked from France".

The announcement by Clinton that the



United States will also push for what he described as a "true-zero" test ban, ends months of argument within the administration about the need for hydronuclear and other low-yield nuclear tests, and comes after a panel of leading physicists and weapons designers ruled that the US does not need such tests to assure the safety and reliability of its nuclear weapons stockpile.

The case for hydronuclear tests, yielding less than four pounds of TNT equivalent through nuclear fission, was studied and rejected by a panel convened this summer under the JASON programme, which provides top-level technical advice to the US government. The panel was chaired by Sid Drell, the Stanford physicist, and included the most senior weapons designers at each of the three US weapons laboratories.

An unclassified executive summary of the full, classified report was released by the Department of Energy last week, after parts of it were referred to in a Senate debate on funding for hydronuclear tests. The Senate voted to appropriate \$50 million for such tests during 1996 — a budget proposal that

the Clinton administration will seek to reverse.

The JASON panel found that "a persuasive case has not been made for the utility of hydronuclear tests" in monitoring the reliability of the weapons stockpile. The tests could help to confirm the safety of the weapons in the event of accidental detonation of one of their explosive charges, the panel said, but "the US arsenal has neither a present nor anticipated need for such reconfirmation".

Hydronuclear experiments are used to obtain information on the fission phase of a nuclear explosion. What distinguishes an HNE from a nuclear test is that the chain reaction is stopped before a full yield explosion occurs. HNEs were developed in the US so that they could continue research during the 1958-1961 test ban, when at least one new weapon was developed using HNEs.

The panel also assessed the usefulness of other types of nuclear tests and experiments that have been proposed as alternatives to full-scale weapons testing. It found that 'reduced yield' tests of up to 500 tonnes TNT equivalent yield from fission could help to ensure stockpile reliability.

But such tests would be useful only if they were continued indefinitely, it said, so the US should not propose them unless it intends to downgrade the Comprehensive Test Ban Treaty to a threshold test-ban treaty. Clinton subsequently announced that the US will push for a ban on such low-yield tests, which some in the Pentagon had wanted to conduct.

But the panel said that so-called hydrodynamic experiments — which use chemical explosives to compress uranium and plutonium, and provide data on the sequence of events between the detonation of a warhead up until the start of the nuclear chain — are useful, and should continue. These are not expected to be covered by any test-ban treaty, because they do not involve a nuclear chain reaction.

Clinton said, however, that the US would reserve the right to withdraw from any test ban on the grounds of "supreme national interest", for example if it ever lacked confidence in a needed weapon. This condition was said to be needed to win support from the joint chiefs of staff, and William Perry, the Defense Secretary for his call for a total test-ban.

Declan Butler & Colin Macilwain



Chirac: turns tables on critics of Pacific tests.

Gene patent study drowned as OTA sinks

Washington. A long-awaited Office of Technology Assessment (OTA) report setting out options for the US Congress on the patenting of human genes will never see the light of day if, as expected, the OTA closes down for good on 30 September.

But two chapters from the original report — on ethics and on technology transfer within the human genome project — will be published next month, as OTA staff scramble to get out what they can before the agency shuts its doors.

The OTA study of the patenting of human DNA sequences began in March 1993 and was originally due to be completed in the summer of 1994. It was requested by Senators Mark Hatfield (Republican, Oregon), Edward Kennedy (Democrat, Massachusetts) and Dennis DeConcini (Democrat, Arizona), after Hatfield had threatened to push for a total moratorium on the patenting of such sequences.

But the issue became less of a priority for Hatfield in the spring of 1994, when the Patent and Trademark Office rejected patent applications by the National Institutes of Health (NIH) for fragments of human DNA sequences (cDNAa), and the NIH stated that it would not appeal against the decision. The OTA board consequently failed to push through the report last sum-

mer, officials say, while the victory of the Republican party in the congressional elections, which put in doubt the future of the OTA, pushed publication back further.

Robyn Nishimi, staff director in charge of the project, has now left the OTA, to work on a Clinton administration investigation into the so-called Gulf War Syndrome. She refused to comment on the project's status.

But Denise Dougherty, a divisional director at OTA, said that two sections of the report would be published next month as briefing papers. The technology transfer issue is of continuing interest to Congress, she said, while Hatfield retains his interest in the ethics issue. But the core of the original project — on patenting, and policy options for Congress to consider — will not be published.

Rebecca Eisenberg, a law professor at the University of Michigan and member of the panel convened by OTA to advise it on gene patenting, says the application of the existing patenting system to gene patents "will

probably sort itself out over time". She believes that no change in the law is needed.

But Jeremy Rifkin, a critic of the biotechnology industry who recently convened a group of US religious leaders to criticize gene patenting on ethical grounds, said it was "ironic" that the report would not appear when "there is more pressure than ever for Congress to define what is patentable and what is not".

The ignominious fate of this study will be shared by many others at the OTA, despite the efforts of its remaining staff. "It's remarkable how hard our staff are working," says Peter Blair, assistant director of the office. "We expect to finish 30 reports before 30 September." About 50 of OTA's 200 staff have left since the beginning of the year.

When it resumes business in September, the House of Representatives will get one last chance to rescue OTA. Amo Houghton (Republican, New York) will put forward a motion to return the Legislative Branch appropriations bill to conference, on the grounds that it does not reflect the House position on the OTA (see *Nature* 375, 711; 1995).

But Robert Walker (Republican, Pennsylvania) says: "I don't hear many people saying they think that resolution will pass."

Colin Macilwain



Hatfield: preoccupied with patent ethics.

Last-minute lifeline for Princeton Tokamak tests

Washington. The US Senate has thrown a desperately needed lifeline to the Princeton Plasma Physics Laboratory (PPPL) in New Jersey, raising hopes that the fusion laboratory will obtain the money needed to continue operating its Tokamak Fusion Test Reactor (TFTR), the main magnetic fusion facility in the US.

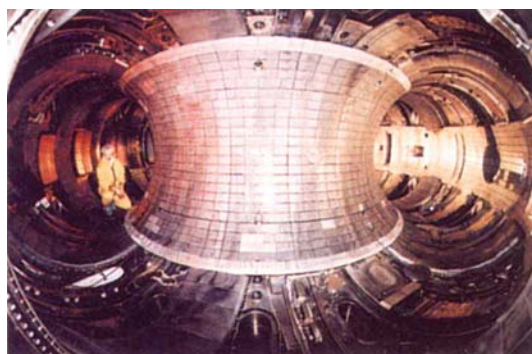
An appropriations bill amendment passed on the Senate floor would allow the Department of Energy (DoE) to spend \$56 million next year to keep TFTR going — provided the department can find the money without cutting other research programmes, for example, by reducing general administrative expenses.

Physicists at PPPL are now banking on the department to make a case to appropriators that it can come up with the money, says Dale Meade, deputy director of the laboratory.

Anne Davies, head of the fusion office at DoE, says: "All of us would very much like to continue to run TFTR. But where we get the money from is problematic." Substantial administrative savings have already been factored into the budget proposals of both the administration and the Congress, she points out.

In February, the administration asked

for \$360 million for magnetic fusion research in the next financial year, which starts on 1 October. The House of Representatives has allowed \$229 million (see *Nature* 375, 622; 1995) and the Senate



Princeton Plasma Physics Laboratory: fusing budgets to keep fusion feasible.

\$225 million, as well as the permission to spend another \$56 million on TFTR.

House and Senate appropriators will confer on 19 September to finalize the Energy and Water appropriations bill. Before then, DoE will appeal, asking them for up to \$320 million for magnetic fusion, and setting out its revised priorities.

For example, the department will drop its plan to build a new Tokamak Physics

Experiment (TPX) at PPPL, and will continue with TFTR instead. The department's revised position, and the delegates' response to it, will seal TFTR's fate.

Recent results from the reactor have addressed the problem of confining plasma in the tokamak by changing the current flux distribution therein. The results "could halve the size and cost" of a commercial tokamak reactor, says Steve Dean of Fusion Power Associates, a fusion advocacy group.

Meanwhile, DoE is seeking to redefine the US contribution to the International Thermal Experimental Reactor (ITER), the other main item in the magnetic fusion budget. At an ITER council meeting last month, the other international partners flatly rejected the idea, proposed recently in an influential report to the US government (see *Nature* 375, 713; 1995), that they should enter negotiations to reduce the scope and cost of the project by two-thirds.

Whatever the final US budget figure for fusion, Davies says that her office will reshape its ITER contribution — in design, research, and basic physics to support the project — in such a way that the partners will accept that it is still contributing "a fair share of the effort".

Colin Macilwain

'Made in Japan' policy jettisoned in space

Tokyo. In a departure from its past policy, Japan's National Space Development Agency (NASDA) will import some components, rather than using only Japanese technology, for a planned upgrade of its H-II launcher to make the rocket cheaper and better able to compete in the satellite launch market.

NASDA's plan to spend ¥65 billion (US\$0.7 billion) over the next five years on developing the upgrade was approved last week by the Space Activities Commission, Japan's principal government body for setting space policy.

At the end of this month, the Science and Technology Agency, which oversees NASDA, will request a budget of ¥2 billion for fiscal year 1996 (which begins in April) to begin development of the upgraded H-II. Such budget requests are very seldom rejected, although the amount may be reduced slightly in negotiations with the Finance Ministry.

The upgrade will be able to place three to four tonnes of payload in geostationary orbit, compared with two tonnes for the current H-II.

Extra lift would be provided by using a liquid fuel LE-7 as a booster — an advanced liquid fuel engine used in the first stage of the current H-II — in addition to the existing two solid fuel boosters. One or two LE-7 boosters would be added depending on the payload. The version with two LE-7 boosters would be used to

launch the planned unmanned space shuttle HOPE.

The decision to import components of the launchers marks a shift in NASDA policy — one goal of the ten-year pro-



Japan's H-II launcher upgrade will be cheaper, more powerful — and part foreign.

gramme to develop the current H-II, which NASDA began in 1984, was to end dependence on US technology by building an all-Japanese launcher. The agency has not decided which components to import, according to one NASDA official, who says

that the matter is "still under study".

At the start of the H-II project, NASDA was confident that the H-II could compete with other launchers in the commercial satellite launch market.

But the yen has since tripled in value against the dollar, and the cost of an H-II launch — around ¥19 billion (US\$204 million) — is around double that of one using Europe's Ariane IV or the United States' Atlas and Delta.

Rocket Systems Corporation, the company created by a consortium of Japanese aerospace companies in 1990 to sell commercial H-II launches, has yet to win a single order.

But while NASDA has set itself the ambitious goal of reducing the cost of a launch by more than half — by using mass production in addition to importing cheaper components — one official from another launcher organization says he is sceptical that such savings can be achieved unless Japan can greatly increase the frequency of launches.

Indeed, NASDA can launch only within two short launch windows annually, in winter and summer, under an agreement with tuna fishermen near its launch centre on Tanegashima island off the coast of Japan's southern island of Kyushu.

Moreover, the government launches only one or two satellites a year and is unlikely to increase this frequency given its budgetary constraints. **David Swinbanks**

Very Large Quake shakes telescope

Munich. A powerful earthquake earlier this month violently shook Mount Paranal, the site in the north Chilean desert of the Very Large Telescope (VLT).

The VLT, which is being built by the European Southern Observatory (ESO), will be the world's largest telescope when it is completed around the end of the century.

But the 8-magnitude earthquake seems to have caused little damage to the enclosure of the first telescope, which is scheduled to be installed next year, according to Massimo Terenghi, project manager of the VLT.

The telescope has been designed to withstand earthquakes of at least 8.5-magnitude.

"The schedule for first light should not be affected", he says, although damage to the shock-absorbing columns will require several weeks to repair.

Since construction began early last year, the building of the VLT has been beset with problems arising from claims by a Chilean family to ownership of the site.

Legal action stopped work for three

weeks in spring last year, but ESO has ignored subsequent embargoes claiming that it has immunity from local law (see *Nature* 373, 551; 1995).

The earthquake comes less than two months after the government stepped in and resolved the issue of ownership in ESO's favour. No casualties were reported at the Mount Paranal site, although three deaths were reported in Antofagasta, the nearest town, which lies 130 km to the south.

According to seismologists, an earthquake of similar intensity can be expected less than once a century in the area.

"Maybe it is good to have happened now, when we have only started the building of the enclosure for the first [of four] telescopes," says Terenghi.

It shows that the engineering design can withstand severe shocks, he says. ESO has offered 25 million (US\$65,000) to assist with reconstruction around Antofagasta, where hundreds of houses were destroyed, says ESO spokesman Roderigo di Castro, "to express its solidarity with the local community". **Allison Abbott**

Japanese science agency loses iron lady

Tokyo. Japan's Science and Technology Agency (STA) last week lost its outspoken and popular leader, Makiko Tanaka, in a cabinet reshuffle.

Tanaka, who is a member of the Liberal Democratic Party (LDP), is renowned for her blunt and straightforward style, which has made her a favourite with the media, and led to STA's public prominence. Her father, the late Kakue Tanaka, was a former prime minister who resigned in the mid-1970s over a scandal about the purchase of Lockheed Tristar aircraft.

Selection of LDP members in the new cabinet was strongly influenced by Yohei Kono, the deputy prime minister who is seeking re-election as head of the LDP and Tanaka's successor, Yasuoki Urano is a soft-spoken consensus builder.

Tanaka's fame peaked earlier this year when she forced the dismissal of an STA official who had opposed her wish to make public the names of semi-government research and development corporations identified as needing reform.

David Swinbanks

Reunification grant shake-up threatens jobs

Munich. Hundreds of scientists in east Germany could lose their jobs in the coming months as government support schemes established after reunification begin to expire. Two-thirds of the 24,000 scientists who worked at institutes of the DDR Academy of Sciences have lost their jobs since reunification in 1990.

Unless there is extra support, "there will be more victims to come, and this is not justified", warns Detlev Ganten, head of the Max Delbrück Centre for Molecular Medicine in East Berlin — one of Germany's 16 national research centres — and a member of the Wissenschaftsrat, Germany's science council.

The job cuts are part of a plan, suggested by the Wissenschaftsrat, to reorganise the institutes along the lines of their Western counterparts. More than DM3.4 billion (US\$ 2.43 billion) has also been spent on modernising the institutes' derelict laboratories and equipment. However, this high level of investment in the east stops this year.

Concern centres on a plan, proposed by the Wissenschaftsrat, to encourage research institutes to rely more on research grants and less on institutional funding. The council argued that by supporting more staff on research grants, institutes would avoid the scientific inflexibility and inertia caused by the practice of providing most scientists with permanent positions.

But the Wissenschaftsrat's target, for institutes in the east to support half their staff on research grants, has not been met. The Deutsche Institute for Nutrition Research (Dife) in Potsdam has filled only 34 of the recommended 120 soft-money positions, while the Institute for Zoo and Wild Animal Research (IZW) in Berlin has managed to fill just two of its target of 50. The Max Delbrück Centre, has been more successful, but, nonetheless, still needs to fill 180 of its 250 soft-money positions.

A DM186.2 million research fund set up in 1992 to provide east German biologists with a safety net while they got to know the Western competitive grants system, has also been reduced. The fund, which originally supported 512 positions, will now cover 118 and expires next year.

This so-called Verstärkungs (boosting) fund "is being brought to an end far too early", says Reinhold Hofmann, director of IZW, where the fund has supported many positions. The Wissenschaftsrat is convinced that the principle of introducing competition remains valid, but now concedes that institutes cannot be expected to fund themselves unless more grant money is provided.

The recession has reduced the amount of grant money available, whereas applicants for grants are increasing. Moreover, the lack of permanent staff, senior scientists or technical support at many institutes means they

often fail to meet the requirement of many funding agencies that an applicant's institute has a sufficient research infrastructure.

East German institutes also find it difficult to qualify for grants from the Deutsche Forschungsgemeinschaft (DFG), which is the main source of research grants in Germany. DFG, which mainly funds university research, only funds projects at research institutes when these differ from the institute's main research direction. But what most East German institutes need is funding for their main research directions.

"To imagine that grant money could support 50 per cent of a research institute is illusory," says Ingolf Hertel, president of the Wissenschaftsgemeinschaft Blaue Liste (WBL), a society that represents the 82 so-called Blue List research institutes in Germany that are jointly supported by the federal and state governments.

The Wissenschaftsrat now argues that the federal government needs to provide more core funding to the institutes to allow the

number of scientific and technical staff to be increased and provide the infrastructure needed to secure funding from grant agencies. Federal and Länder governments should make more research grant money available, it adds.

The Wissenschaftsrat also argues that the ministry of education and research (BMBF) should extend current levels of research spending in the east; BMBF spends DM190 per capita on education and research annually in the east — a total of DM3 billion — compared with DM165 in the west. The DFG should also make more funds available to non-university researchers, it says.

BMBF insists, however, it will not provide extra funds for research in the east, arguing that the five-year reorganisation has resulted in "a nearly complete harmonization of the research landscape" between west and east. Additional funding of institutes in the east would have to be at the expense of other research activities in Germany, it says.

Quirin Schiermeier

Sacked chimpanzee centre director complains of monkey business

Washington. New York University Medical Center has fired Jan Moor-Jankowski, the director of its chimpanzee facility, the Laboratory for Experimental Medicine and Surgery (LEMSIP) at Sterling Forest, New York, and donated the laboratory to the Coulston Foundation of New Mexico.

Moor-Jankowski claims, however, that he is being victimised by NYU for having criticised animal experiments carried out at other NYU facilities. He has threatened the university with court action.

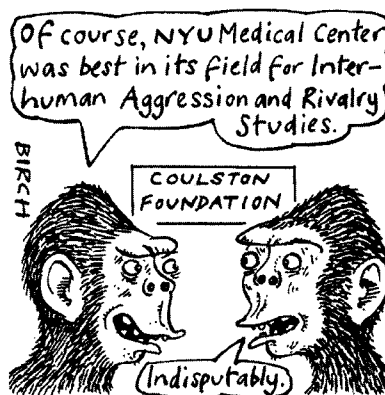
In 1991, Moor-Jankowski won a libel battle against Immuno, the Austrian pharmaceutical company, which concerned criticisms of the company's collection of chimps in Africa published in the *Journal of Medical Primatology*, which he edits. Moor-Jankowski says that Frederick Coulston, the toxicologist who runs the Coulston Foundation, was named as a "friend of court" supporting Immuno in that case.

In a statement, David Scotch, associate dean of the NYU School of Medicine, said that in transferring LEMSIP to the Coulston Foundation, "our primary concern is and has been the welfare of the chimpanzee population at LEMSIP".

Moor-Jankowski says he has now been locked out of LEMSIP. "Dr Scotch has placed a security guard to prevent me entering the laboratory which I founded," he says. "I'll sue them as a member of faculty who has been discriminated against and harassed."

He alleges that the university only moved to dispose of LEMSIP after he had complained at meetings of NYU's animal care committee about the use of chimpanzees in experiments with crack cocaine at the university's department of environmental medicine.

The US Department of Agriculture is



investigating these complaints, and on 8 August Patricia Jensen, an assistant secretary at the department, wrote to L. Jay Oliva, president of NYU, suggesting that he delay the sale of LEMSIP until the investigations were complete.

Dan Perkes, head of communications at NYU, said that the letter was received after papers relating to the transfer of LEMSIP had been signed, but before NYU announced it on 9 August. Colin Macilwain

OSPREY makes waves in UK energy research

London. The launch of the world's first commercial wave-power station in Scotland earlier this month may reopen the debate about the wisdom of the British government's decade-long suspension of long-term funding for wave energy studies.

The £1.9 million Ocean Swell Powered Renewable Energy generator (OSPREY) has been developed by the Inverness-based company Applied Research and Technology Ltd. The company raised £435,000 (US\$696,000 of the costs needed from the European Union's Joule non-nuclear energy research programme, and the remainder from private companies including AEA Technology, Scottish Hydroelectric and GEC Alsthom. British Steel donated 800 tonnes of steel to the project.

OSPREY, weighs 8,000 tonnes, took five years to develop and is expected to operate for 25 years. It will be installed off the coast of Dounreay, northern Scotland, and will generate 2MW of electricity, enough power for 2,000 households. Electricity will be carried to land via an undersea cable. A wind turbine carried on the vessel can generate an additional 1.5MW.

The British government provided £50,000 for water-tank tests of an OSPREY model, but since 1983 has stopped all support for research into wave energy on the grounds that its high initial capital costs would make it uneconomic.

IMAGE
UNAVAILABLE
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REASONS

Ruling the waves: Will Osprey turn the tide in favour of UK wave-energy research.

The government's position was endorsed in 1992, when a feasibility study commissioned from the Department of Trade and Industry's (DTI) Renewable Energy Advisory Group concluded that wave generators "would be unlikely to generate electricity competitively in the short to medium term".

The study's findings were taken up by a review commissioned last year by the DTI, which recommended that the government should maintain the freeze on funding. Tim Eggar, the minister for energy, subsequently confirmed, in a written reply to a question in the House of Commons, that no money would be available for wave power, which he said had "limited potential to contribute commercially to energy supplies".

David Ross, an environmental journalist,

and author of a forthcoming book *Power from the waves*, disputes the government's conclusions: "How can wave power be considered expensive?" he asks. "All the costs are in the beginning, after that, it is free." The United Kingdom has 12,000MW of potentially usable wave power, equivalent to the installed generating capacity of Scotland. According to Ross, "wave-power research was sacrificed because the government wanted to develop nuclear power. It was a political decision".

Meanwhile, researchers at the University of Edinburgh have won an ECU800,000 (US\$1.05 million) grant from the Joule programme to develop a hub for a two-tonne turbine blade for a wave generator destined for the Azores islands in the Atlantic Ocean. Stephen Salter, professor of engineering design at the university, says the development of OSPREY represents "a huge loss of face" for the British government. "It is a considerable achievement to get private money in the face of opposition from your own government."

The OSPREY project, Salter believes, represents a milestone in renewable energy research. "It has the same significance as the Blériot flight across the English Channel," he asserts. "Few at the time believed that air travel was possible, fewer still were prepared to back the project. But it still succeeded."

Ehsan Masood

Corn crosses last hurdle for genetically modified crops

Oxford, UK. The US Environmental Protection Agency (EPA) has approved applications from two companies to market for the first time corn seeds genetically engineered to produce pesticides against the European corn borer, a pest that causes losses of around US\$1 billion annually to farmers in the United States.

The approval, granted to the US agricultural biotechnology company Mycogen Corporation and the Swiss company Ciba Seeds, clears the way for commercial use by next year of the transgenic corn seeds. These contain a gene from the soil bacterium *Bacillus thuringiensis* (Bt) that produces a protein that is toxic to insect pests such as the European corn borer.

This EPA approval is the final regulatory hurdle for the modified corn, as the US Food and Drug Administration has cleared it on safety grounds. The US Department of Agriculture, meanwhile, says it will treat genetically modified corn seeds in the same way as corn hybrids bred by conventional means.

Advocates of agricultural biotechnology argue that such modified hybrids will reduce or eliminate the need for conventional chemical insecticides. The comp-

IMAGE
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REASONS

Field day: Genetically-engineered corn will eliminate the need for chemical insecticides.

anies expect Bt-modified seed to capture a significant share of the \$2-billion US seed-corn market.

Bacillus thuringiensis is not a stranger to pest control. Organic farmers have been spraying it on crops as a natural pesticide for several decades. The Union of Concerned Scientists is worried that constantly exposing corn borers to Bt at the levels at which corn is grown will increase the likeli-

hood that the pests will develop resistance.

Mindful of these concerns, the EPA has included conditions in the approval ordering the companies to monitor insect resistance, and to conduct research to develop resistance management plans and to develop plans should the insects become resistant.

Jerry Caulder, chief executive of Mycogen, says the likelihood of increased resistance has been reduced because the company's transgenic corn seed contains both natural and Bt-based control genes. This combination, he says, is a key element of a resistance-management programme his company has developed to protect Bt's long-term effectiveness in crop protection.

The EPA approval does not open the door for biotech corn-on-the-cob or popcorn just yet, however, as the EPA registration is limited to commercial field corn production. It is possible that some modified corn might be used in the production of high-fructose corn syrup, a sweetener used by the soft drinks industry. EPA officials will evaluate the companies' compliance with registration requirements and decide whether to allow full commercialization in five years.

Mike Ward

Bad manners, not good

SIR — Your leading article “Good manners win out” (*Nature* 375, 2; 1995) is, in my opinion, misleading. When grants are awarded by the US Public Health Services, they are awarded to the institution, with responsibility for the work being delegated to the principal investigator. Responsibility for governing access to and publication rights over such research traditionally rests with the institution.

After reviewing the history of the Irish schizophrenia studies to which you refer, it is my opinion that the study was primarily the work of Dr Kenneth Kendler, although Dr Scott R. Diehl clearly made an important contribution. Kendler, who devised these studies and began them before Diehl's arrival at Virginia Commonwealth University (VCU) should, in my opinion, be a significant author on major papers from this study. Clearly, papers generated by a team of investigators should not be published by a junior member of the team without the inclusion of the team leader.

Shortly after they became aware of the initial findings of linkage to schizophrenia on chromosome 6p in the spring of 1993, Kendler proposed that Diehl should write a paper reporting these results, with Diehl as first author, Kendler as last author and other Irish and VCU investigators as co-authors. Diehl refused this offer, as he did again towards the end of 1994.

The National Institutes of Health (NIH) were never asked by us to adjudicate the issue of access to and publication of the Irish studies. Indeed, the National Institute of Mental Health, the part of NIH that funded the work of Kendler and Diehl, specifically rebuffed Diehl's attempts to gain access to these data, stating that it was VCU's responsibility to decide the matter. Diehl did issue an ultimatum to his colleagues that if we did not agree to his publication plan (which included S. Wang as first author and Diehl as last author), he would publish the results on his own. Both I and the leading Irish collaborator on the project, Dr Dermot Walsh, told Diehl that such action would be inappropriate.

Your choice of title, “Good manners win out”, is particularly ironic. Kendler has spent more than ten years building a collaboration with his Irish colleagues and they have collected the largest and most informative linkage study of schizophrenia yet available. Diehl published these data without the permission or knowledge of Kendler, his Irish colleagues or this institution, where the work was carried out. Whereas Kendler, following standard scientific etiquette, kept Diehl informed of his publication plans, Diehl prepared and submitted his manuscript in secret. Kendler has worked scrupulously to

ensure proper academic credit to his Irish colleagues, including several young Irish psychiatrists who spent years working on this project, while Diehl's preemptory publication has deprived them of appropriate recognition. A more appropriate title for your leading article would have been “Bad manners win out”.

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■ The alternative title suggested is much better — Editor, *Nature*. □

Science in India

SIR — Universities have a profound role to play in generating knowledge, as well as transmitting that knowledge from one generation to the next. The responsibility to do this rests squarely on faculty members. At leading UK universities, such as Oxford, Cambridge and Glasgow, it is clear that university lecturers have a distinct role to play in enriching the lives of those they teach. And what is taught must stand up to the scrutiny of the students. It is good quality research that makes the lecturer acceptable to the system.

So what has gone wrong in Indian universities? Financial difficulties, student unrest, non-academic influences both inside and outside the universities all play a part. But none is the root cause of present problems. I am optimistic that things may improve and should like to suggest a remedy.

The most severe blow to academics in Indian universities has resulted from the rapid growth in numbers of so-called professors benefiting from merit promotion schemes that have been arbitrarily applied. The problems will continue if the process is not effectively monitored and reversed. These upstarts create all sorts of problems for younger scientists. For example, they demand co-authorship of research papers for which they hardly deserve even an acknowledgement. This obviously helps those young researchers who agree to co-authorship in the expectation of favours from their superiors.

As professors are usually members of decision-making committees, active researchers can be effectively silenced. And eminent academics from the Indian Institutes of Technology and the national laboratories join these professors in approving PhD theses in which they have taken no part. Similarly, Indian scientists working abroad connive in this process in order to obtain opportunities to visit Indian institutions. A young scientist working abroad after gaining a bachelor's degree in engineering in India has been known

not only to act as an examiner of a PhD thesis in physics but also to approve it within a fortnight from the date of despatch as if he were anticipating it. Thesis supervisors take undue credit for the work of their protégés. Is this not a form of prostitution?

My belief is that this whole process is the principal cause of the problems in Indian universities. Unproductive academics of the kind I have described outnumber other researchers and are able to humiliate and embarrass them in many ways.

My remedy is to propose that all faculty members should take part in appointing and promoting staff to ensure that people are accountable for their actions. For myself, I have been in academic life for 15 years, having rejected an offer to work with an eminent astronomer in order to take part in academic life, and I love teaching. But I would have welcomed an opportunity of being evaluated for promotion on the basis of my research.

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Effects of DDE

SIR — A leading article in *Nature*¹ states that “the major metabolite of DDT, *p,p'* DDE, is a demasculinizing agent”, and therefore “the case for research to be conducted urgently is overwhelming”. It is unlikely that such research could be conducted with human subjects, but information is available on 63 human males exposed to DDT (and hence to DDE) in the DDT manufacturing plant in Torrance, California, with a median of 15 years of exposure². No cases of cancer were found during 19 years of employment. The married male workers averaged four children per family. “The largest families had as many as 13 children” and the (male) supervisor had 8 children (personal communication).

The effects of DDE, 100 mg per kg per day, on rats described by Kelce *et al.*³ corresponded to 7 grams of DDE per day for a 70 kg human. This is quite disproportionate to levels of DDE, median about 10 nanograms per ml of blood, found in humans⁴. Lower levels of DDE had no effect on reproduction in rats or beagle dogs^{5,6}.

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Drugs in ancient texts

SIR — Bart K. Holland has argued for less random screening of plants in the rain-forests and suggested more studies of ancient Western medical literature in the search for new drugs¹.

We would like to draw attention to some studies where ancient texts have been investigated. A most conscientious study was undertaken by Hartwell at the US National Cancer Institute (NCI) in the 1960s. Hartwell systematically searched several hundred medical texts dating back as far as 2800 BC including the classical writings of Greece and Rome, for plants used against cancer. The names of more than 3,000 plant species were recorded². One problem in dealing with ancient texts is plant identification, another is the interpretation of the diseases. Nevertheless, 19.9 per cent of the plants used against cancer in ethnomedicine had positive results in the antitumour screening performed by the NCI, compared with 10.4 per cent obtained from random screening³.

Discussing the history and future of ethnopharmacology, Holmstedt has called attention to the ironic history of ephedrine, a well known 'modern' drug⁴. Ephedrine had been used in China for more than 5,000 years as the crude drug *ma huang* before it was introduced in modern science in the 1920s. In 1975, Majno⁵ reported finding a reference to a plant called *ephedron* in the writings of Pliny (AD 23–79). The plant was used to stop bleeding and treat coughs, as ephedrine is in today's medicine. There are several European *Ephedra* species, so Pliny is probably dealing with an independent Mediterranean discovery.

We have a project dealing with Swedish traditional medicine with emphasis on written sources from the fifteenth to the nineteenth centuries. Among these books and dissertations are Linnaeus's vast production, those of other well reputed Swedish eighteenth century doctors and medieval handwritten sources. This literature consists of approximately 50 books and a great number of dissertations, mainly from the eighteenth century. Information on plants used to treat inflammation was extracted from the old texts and these plants were evaluated in biological tests⁶. Active plants are being studied to determine their active constituents.

In *Materia Medica* of 1749 (ref. 7), Linnaeus suggested the use of *Menyanthes trifoliata* L. in the treatment of nephritis and rheumatism. A decoction of this plant is still used to treat glomerulonephritis in Swedish ethnomedicine. We studied its effect in a model for renal failure, inducing an inflammation. Rats treated with the decoction had a three times higher glomerular filtration rate after ischemia

than the control group⁸. We are now evaluating the pharmacological properties of the decoction and isolated bioactive compounds.

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Italian research

SIR — The recent interview with Romano Prodi on Italian research, and the comments on the National Research Council (CNR) in particular (*Nature* **375**, 620–621; 1995), requires a response to the statement about CNR being throttled by money "going on salaries rather than research programmes".

CNR not only provides funds for the academic community but also has its own scientific network with about 2,500 full-time scientists. I have served on a CNR central advisory board for the past ten years, and to my knowledge salaries — most of them for those in the scientific network — account for less than half the annual budget. This is less than in many industries, and in universities salaries account for more than 90 per cent of the budget. Funds for CNR and basic research are about 20 per cent of the budget, and funds for applied research administered by CNR between 15 and 20 per cent. (Only 10–15 per cent of this goes to CNR researchers, while 50 per cent goes to industry.)

What has actually been increasingly throttling CNR for the past decade is that 15 per cent of the budget is devoted to accommodation, maintenance, security and other nonscientific costs. Of the money reaching the CNR network of more than 300 institutes and centres, about 50–70 per cent is spent on running costs. The amount available for each CNR scientist for research is therefore less than ECU5,000 a year. But it may be for the Italian courts rather than the scientific community to consider this anomaly.

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Origins of Frazer's Golden Bough

SIR — I believe I have found the original source of Frazer's *Golden Bough* in two essays by the French scholar Henri Gaidoz. This is not recorded in the current literature or in R. Ackerman's beautiful book (*J. G. Frazer: The Man and His Work*, Cambridge, 1897).

Gaidoz was a student of Celtic religion and wrote *La religion gauloise et le gui de chêne* (Leroux, Paris, 1880) and *Deux parallèles: Rome et le Congo* (*Revue de l'Histoire des Religions* **VII**, 5–16; 1883).

Gaidoz is interested in the two *topoi* of the golden bough and Nemi's priest, which are the *Leitmotiv* of Frazer's speculation. The Scottish anthropologist probably never directly read Gaidoz, but was influenced by Ernest Renan's philosophical drama, *Le prêtre de Nemi* (1885), and Renan was surely acquainted with Gaidoz's writings.

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New world, old word

SIR — You refer (*Nature* **375**, 730; 1995) to a meteorological event in Israel that caused cool air "to collapse towards the cacti at high speed".

You may be confusing Israel with another country in the Western Hemisphere, as the Cactaceae are endemic to the New World and are not usually found in the Old. A few species of *Rhipsalis* occur in East Africa, Madagascar and Sri Lanka, but there are none in the Middle East.

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■ Declan Butler writes: I am afraid that alliterative licence got the better of me. □

SIR — In "At home in Zion" (*Nature* **375**, 720; 1995), R. Boxman is quoted as saying that the Hebrew neologism for electricity, *hashamal*, amber in Hebrew, was chosen after the amber arcs in the clouds seen by Ezekiel during a vision.

Although I do not speak Hebrew, I believe there was another reason for this choice. The origin of the international word 'electricity' is the Greek name for amber, *elektron*. It was the physicist and physician to Queen Elizabeth I of England, William Gilbert of Colchester (1544–1603), who, in his great work *De Magnete*, published in 1600, called bodies that attract in the same way as rubbed amber "electric" and the attracting force "vis electrica" (amber force).

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In praise of interdisciplinarity

Overspecialization in science is a bane of the times, but a meeting last week showed the benefits to be had from reversing the trend.

Keystone, Colorado. Whatever happened to the scientific generalist? Half a century ago, the physicist George Gamow felt able to include a discussion of modern cell biology and genetics in his popular book *One Two Three ... Infinity*; it would be a brave physicist who would make such an attempt today. Although general popularizers of science do exist (and are good at their job), they are not of the stature of Gamow. With few exceptions (such as semi-retired 'elder statesmen' or workers in explicitly interdisciplinary fields), our top-notch scientists seem to live in a more rarefied atmosphere — feeding, if not on a diet of pure Higgs bosons or synaptic vesicles, then at least on undiluted physical or biological science.

The reasons for this increasing specialization are apparent. First, as more people have been occupied in scientific research this century, and have been communicating more readily with one another, the accumulation of new understanding has proceeded ever more quickly. Although successful textbooks have always had several editions, in some fields there is now talk of updating university-level texts every year. Scientists who last studied biology twenty years ago no longer have even the appropriate vocabulary to understand many current advances in molecular biology — a problem with which this journal wrestles each week.

At the same time as scientific understanding has been advancing more rapidly, scientists have had less time to indulge in perceived luxuries such as keeping up with other fields. The pressures are familiar ones: more time spent on administration, on acquiring funding, and on helping students to survive in an increasingly competitive environment, to mention a few. Moreover, the easiest way to succeed in the competition for funding and university positions is to become expert in a single field of research; jacks (and jills) of all trades invariably lose out to masters of one.

Is specialization necessarily a bad thing? Surely it is more efficient for scientists to specialize, just as it makes sense for musicians to decide at an early age whether to be violinists or tuba players: the skills required are different in detail, and take years to develop. But an orchestra plays more beautifully if its members are sympathetic to each other's roles, and one can argue that, especially in times of reduced funding for research, a similar sympathy between practitioners is necessary for the scientific enterprise to flourish. Advocates of the Superconducting Super Collider project,

who failed to convince the US Congress to commit \$11 billion to the promise of finding "new physics" at energies in excess of 10^{13} electron volts, no doubt rue the fact that they were not first able to enlist the support of their fellow scientists — in some cases (though not in all) because the advocates were not able to explain themselves sufficiently well.

Against this background, the Keystone Center — a non-profit organization known to many readers of this journal for its symposia on molecular and cellular biology — inaugurated in 1991 an almost-annual series of unashamedly interdisciplinary meetings, known as the Scientist to Scientist colloquia. This year's colloquium, held last week in Keystone, treated about 70 scientists to talks on fields ranging from the molecular basis of memory to the detection of galactic 'dark matter', and from dinosaur behaviour to particle physics. With only fifteen 45-minute talks in four and a half days, there was plenty of time for discussion — which never ran dry. Scientists, young and old, who had evidently not had such concentrated exposure to ideas outside their own fields since their undergraduate days, grew visibly more excited as the week unfolded, as they rediscovered the pleasure of learning for its own sake. For as much as the colloquium series may have a pragmatic purpose — to give scientists the wherewithal to hang together (in the words of Benjamin Franklin), so as not to hang separately — its organizers are equally aware that the meetings provide participants with a less directed but equally tangible benefit: namely, the sheer inspiration of seeing how clever other people are.

Put another way, the meeting is simply great fun. For example, neuroscientists and astrophysicists alike seemed to delight in the mutual discovery that there are about as many neurons in the human brain ($\sim 10^{11}$) as there are stars in our Galaxy — not a profound realization, but one that gave each practitioner a better feeling for the other's work. At the same time, a computer scientist discovered, from a talk on synaptic transmission, that the most powerful computers are already matching the human brain in number of switching events per second — a graphic illustration that much remains to be learned about the efficient use of computational resources. And who is to say which is more wonderful: the fact that one can now record simultaneously the activity of 150 neurons in the hippocampus of a rat going about its business (and then apparently replaying the day's events during sleep, for

the benefit of the neocortex), or the existence of a Cretaceous fossil from Mongolia, which shows a *Velociraptor* using its arms to grasp the skull of a *Protoceratops*, while its killing claws are buried in the hapless victim's chest? Indeed, the participants' respect for their peers' resourcefulness in prising out nature's secrets was surpassed only by their wonder at the character of the natural world thus revealed.

Do meetings of this type yield more than a week of delight for 70 scientists? This is a pressing question for the meeting's organizers, who have had some trouble in finding sponsors for the series. Judging from this year's example, however, there are certainly benefits to be had beyond the re-energizing of the immediate participants (which is itself of some value). Although governments these days may be less universally keen to avail themselves of scientific advice than once was the case, leading scientists are still expected to be able to offer advice on technical matters, often outside the scientist's own field. As some of this advice will concern the relative importance of research in different fields, it would seem essential for the advisers to expose themselves to the kind of continuing education offered by the Keystone colloquia. In this regard, it is to be welcomed that two of the participants in previous colloquia have successfully replicated the Keystone model in their own universities. It is also a shame that only a very few university administrators and funding-agency officials have accepted invitations to Keystone.

Another important product of the colloquia, wholeheartedly welcomed by this journal, is a growing collection of scientists who are able to communicate the interest and excitement of their work in an accessible fashion. The meeting's organizers make a great effort to choose speakers who eschew jargon, and the audience is encouraged to request clarification on the spot where necessary. (This year a particle physicist interrupted a biological chemist with "What's a pi-orbital?") It is a tribute to the spirit of the meeting that the onus was on the speaker to explain, rather than on the physicist to have been better informed.) If the Scientist to Scientist colloquia manage to attain the prestige that they deserve, we may hope to see the day when leading scientists compete with one another for clarity of expression, rather than simply for intellectual attainment within their chosen field. That is a consummation devoutly to be wished.

Laura Garwin

RNA seeks its maker

Joseph A. Piccirilli

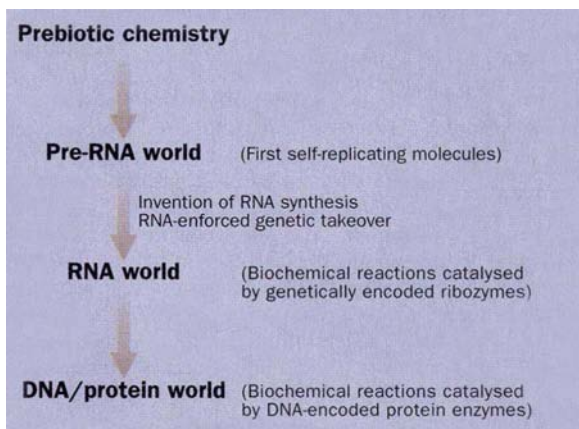
THERE is plenty of evidence that catalytic RNA arose early in the evolution of life on Earth¹. But was RNA involved from the beginning? Given that the experimental synthesis of ribonucleotides and non-enzymatic replication of RNA under plausible prebiotic conditions has proved to be so difficult, it seems the answer may be 'no'^{2,3}. Instead, life may have begun with some simpler replicating system that was later replaced by RNA, and on page 578 of this issue⁴ Böhrer, Nielsen and Orgel describe the first clues that such a system could have fallen prey to genetic takeover by RNA. They show that a polymer consisting of a protein-like backbone with nucleic acid bases for side chains can, like RNA, participate in template-directed molecular assembly reactions.

According to those familiar with the issues in prebiotic chemistry^{2,3}, it is unlikely that an abundant pool of RNA building-blocks, the β -D-ribonucleotides, existed on the prebiotic Earth. Although the components from which the nucleotides might be synthesized — heterocyclic bases, ribose sugar and polyphosphate — are produced relatively easily under prebiotic conditions, attempts to assemble them under such conditions have met with only modest success. Yields of purine nucleosides (adenosine and guanosine) have been encouraging, but formation of pyrimidine nucleosides (cytidine and uridine) occurs with very low efficiency. Moreover, ribose, which is produced through a series of reactions of formaldehyde, is always a minor product in a complex mixture of sugars. So even if there were efficient reactions for nucleotide formation on the prebiotic Earth, many related molecules having incorrect structures, including stereoisomers, would have accompanied the natural nucleotides.

Pursuit of self-replicating RNA systems using non-enzymatic, template-directed polycondensations of mononucleotides has also revealed major obstacles². The distribution of the natural 3'-5'-linked and the unnatural 2'-5'-linked regioisomers is sensitive to metal ions, and the identity of the monomers and template, as well as the leaving group at phosphorus. Another severe limitation is that purine-rich templates are unable to direct the oligomerization of their complementary

pyrimidine nucleotides, thereby preventing completion of an entire replication cycle. There is also the problem of enantiomeric cross-inhibition — oligomerization is inhibited in the presence of equal numbers of right-handed and left-handed nucleotides³.

All in all, then, it is difficult to conceive of the steps by which self-replicating RNA might have emerged on the primitive Earth; nonetheless, the catalytic and informational properties of RNA, together



Possible stages in the origin of life on Earth. Experiments suggest that chemistry under the conditions of the prebiotic Earth could not have given rise to self-replicating RNA. Instead, a simpler self-replicating informational molecule, possibly like PNA, is believed to be the predecessor of the RNA world.

with the importance of RNA and RNA co-factors in extant life forms, strongly point to the existence of an RNA world¹. Models for the origin and evolution of life must therefore identify informational molecules capable of self-replication that could have arisen on the prebiotic Earth, and must include some mechanism for genetic takeover⁵ — that is, a pathway by which the RNA world came into being (see figure).

Foremost among the candidates for such molecules are 'simpler' genetic materials constructed from flexible, acyclic, achiral nucleotide analogues^{2,6}. One type of nucleoside analogue that fits that description is peptide nucleic acid (PNA), first designed and synthesized as an antisense agent⁷⁻⁹. PNA consists of a peptide (polyamide) backbone composed of *N*-(2-aminoethyl) glycine units to which nucleobases are attached by carbonyl methylene linkers (see figure on page 579 of this issue). Although PNAs may not be prebiotic, their known propensity to pair with DNA and RNA according to the Watson-Crick base-pairing rules suggests a good starting point for the evaluation of the possibility of genetic

takeover, and sets the stage for the current work.

Böhrer *et al.*⁴ report that a polycytidine decamer of PNA (PNA-C₁₀) can act as a template for the oligomerization of activated guanosine mononucleotides in the presence of sodium and magnesium ions. They find that the PNA template is not as efficient as the corresponding DNA template, but still markedly accelerates the condensation of guanosine over the template-independent reaction. Presumably a complex between the template and reactants is formed, involving Watson-Crick base pairing which brings together the activated 5' end of one reactant and the free 2'(3') end of another, facilitating the formation of an intermolecular bond.

The initial phase of this oligomerization process results in the production of dinucleotides and trinucleotides with the non-natural 2'-5' linkages predominating over the natural 3'-5' phosphodiester linkages. However, subsequent elongation steps appear to be specific for 3'-5'-linked oligonucleotide 'primers' and occur in a highly regioselective fashion, yielding 3'-5' phosphodiester linkages. These results imply that a primitive genetic polymer could have transcribed its detailed genetic information directly to RNA to initiate the RNA world.

In a kind of reverse transcription process, the authors also show that a cytidine decamer of DNA (dC₁₀) can direct the ligation of guanosine PNA dimers. This multidirectional flow of information hints at the possibility that the replication of informational molecules on the primitive Earth may not have been self-contained and may have involved coevolution of multiple genetic systems. Finally, in a process that would be equivalent to the first step of self replication, PNA-C₁₀ showed a modest ability to direct the oligomerization of guanosine PNA dimers forming a complementary strand.

Taken together, these results embody the type of subreactions that might be necessary for an informational molecule to achieve self-replication and eventually undergo metamorphosis to a ribose-phosphate backbone. It remains to be seen whether PNA-like molecules are truly prebiotic, and whether template-driven

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events involving them suffer from the same discouraging limitations as analogous processes with RNA. And, of course, we are still left with the problem of ribonucleotide synthesis. Could the ancestor of RNA have folded into catalysts that generate β -D-ribonucleotides? Many details remain unclear.

Perhaps the most significant implication of the new work is that it demonstrates a link between informational polymers of contemporary organisms and those previously considered to be inappropriate for

prebiotic studies, thereby greatly expanding the repertoire for re-enacting life's beginnings. Suddenly, the gap between the anti-RNA conditions on the primitive Earth and the RNA world does not appear so forbiddingly wide. $\square$

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NEUROPSYCHOLOGY

Is dopamine a missing link?

Robert Desimone

THE ability to grab a fleeting item of information coming through our sensory systems and to hold it on-line for a few seconds while we plan a response is one of the defining features of working memory. Work in non-human primates indicates that this aspect of working memory is mediated, at least in part, by 'voluntarily' maintaining the neuronal response to a stimulus for up to many seconds after the stimulus is no longer present. In a paper on page 572 of this issue, Williams and Goldman-Rakic¹ now show from combined physiological recording and iontophoresis of drugs into monkey prefrontal cortex that this deceptively simple mnemonic process is regulated by the D1 dopamine receptor. This provides a potential mechanistic link between four of the most commonly associated subjects in mental health research, namely, prefrontal cortex, working memory, dopamine and neuropsychiatric disorders.

The notion of working memory comes from human cognitive psychology, where it encompasses a variety of interrelated cognitive phenomena and concepts, including covert articulation or rehearsal of verbal material, visual imagery, active short-term memory, and the actions of a 'central executive' that coordinates and plans behaviour and participates in problem solving². All of these processes involving the maintenance and manipulation of information are distinguished by the fact that they seem to be under voluntary, conscious and effortful control. Considering that working memory is integral to cognition in general, it is not surprising that it is disturbed in many neuropsychiatric disorders, such as schizophrenia. The dopaminergic system has been implicated in several such disorders, and pharmacological treatments involving the dopaminergic system are known to affect working memory in both normal people and psychiatric patients. Brain-imaging studies measuring cerebral blood flow have shown that prefrontal areas are selectively activated when nor-

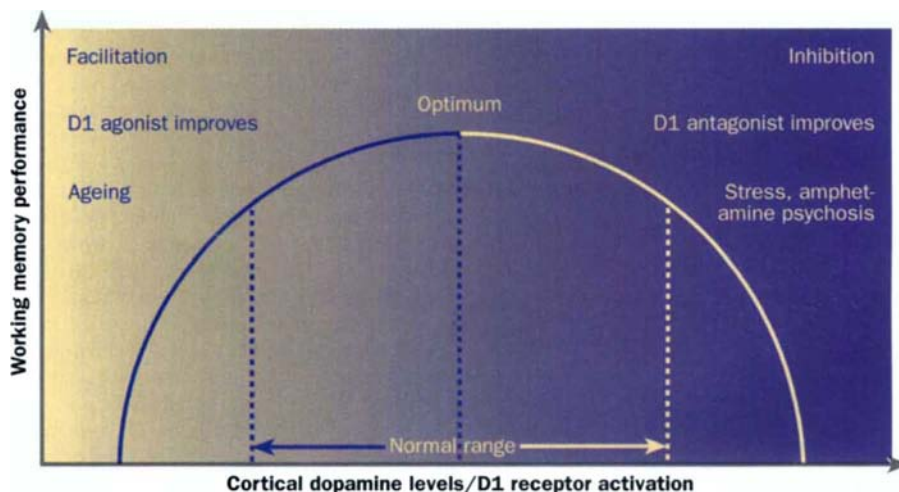
mal people perform tasks requiring working memory, and this prefrontal activation seems to be abnormal in patients with schizophrenia³.

The physiological evidence for working memory in animals has typically come from studies in which animals are given a brief cue to hold in memory during a delay period of a few seconds and are then required to make some choice or response based on this cue. More than two decades ago physiological studies in monkeys found that neurons in prefrontal cortex become activated during the delay period of such a task^{4,5}, with different neurons showing different levels of activity depending on the cue⁶. That is, prefrontal cells seem to maintain a representation of the cue during the delay. This delay activity seems to be under the monkey's voluntary control, as it occurs only for stimuli that the monkey is required to remember and terminates as soon as the memory is no longer required. Since then, studies have shown comparable delay activity in pre-

motor cortex, parietal cortex, visual cortex and several subcortical structures including the striatum, with the anatomical locus of the effects depending on the type of information represented⁷. Despite the widespread distribution of cells with such properties, prefrontal cortex may be a critical site or even one of the originators of the signals that activate cells in other areas. In inferior temporal cortex, for example, delay activity is disrupted whenever the animal processes intervening sensory inputs, whereas it is maintained in prefrontal cortex as long as the animal is able to maintain the memory of the cue^{8,9}. Furthermore, local cooling of prefrontal cortex greatly diminishes delay activity of neurons in posterior parietal cortex during spatial working memory tasks¹⁰.

In their new study, Williams and Goldman-Rakic used a spatial delayed response task, in which the monkey was trained to remember the location of a cue flashed on a screen and to make an eye movement to that location after a delay period. Some cells in prefrontal cortex were activated by the cue and maintained their activity during the delay, but only when the cue location fell within a certain portion of the screen, which is termed the 'memory field' of the cell. When the authors iontophoresed an antagonist of the D1 dopamine receptor at low currents, the delay activity was enhanced only for cues presented in the memory field of the cell; no other aspect of the cell's firing rate was changed. Thus, the signal-to-noise ratio of the cell was increased by the D1 blocker, just for the mnemonic information in the task.

This was a very surprising result, as it had previously been shown that D1 antagonists injected either systemically or directly into the prefrontal cortex actually impaired the behavioural performance on a working-memory task¹¹. An explanation emerged when Williams and Goldman-



An inverted 'U'-shaped function for the role of the dopamine D1 receptor in prefrontal mechanisms for working memory. When either prefrontal dopamine levels or D1 activity are below the optimal range, as may occur with ageing, or above the optimal range, as may occur in stress and amphetamine psychosis, working memory performance is impaired. In such instances, D1 agonists or antagonists, respectively, may restore performance to the optimal range.

Rakic tried higher injection currents: the higher concentration of the antagonist nonspecifically inhibited the firing of the cells. They found the same nonspecific inhibition on injecting any amount of a D2 antagonist. The regulation of prefrontal activity by dopamine is thus more complex than could have possibly been imagined. The exquisite sensitivity of cells both to the magnitude of the receptor occupancy and to the specific dopamine receptor contacted would explain why alterations in either the amount of dopamine in the synapse or in the activity in one of the receptors so often lead to working memory impairments. Conversely, the results open the possibility for improving working memory function through carefully balanced receptor manipulations.

Is the D1 receptor the tie that binds together working memory, prefrontal cortex and neuropsychiatric disease such as schizophrenia? If so, there must be many other links in the chain. Typical neuroleptic drugs that improve schizophrenic symptoms work primarily through the D2 receptor, not the D1, and the more recently developed atypical antipsychotic drugs have much more affinity for a subtype of 5-HT (serotonin) receptor than for either D1 or D2 receptors¹².

Probably the safest answer to the question at this point is that the dopaminergic system does not work in isolation in the cortex but rather interacts in a complex fashion with other modulatory systems. These interactions will have to be worked out at the physiological, pharmacological and behavioural levels, using the delayed response task and other tasks in monkeys that tap some of the more complex aspects of human working memory. Indeed, one of the most exciting aspects of the study by Williams and Goldman-Rakic is that single-cell physiology and pharmacology can now actually be accomplished in awake monkeys performing tasks that engage cognition. □

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Gadolinium peanuts

P. J. Twin

THE recent observation of γ -rays characteristic of a hyperdeformed or 'peanut-shaped' nucleus in the rare-earth element gadolinium-147 has excited nuclear structure physicists. The experiment, reported in *Physical Review Letters*¹, was carried out by a collaboration working on the large multi-detector γ -ray array Gammasphere at the Lawrence Berkeley Laboratory in California.

Nuclei have a spherical or slightly prolate deformation in their ground state, but much larger deformations become energetically possible in some nuclei as they rotate at large angular momenta. Superdeformed nuclei (in which the major axis is twice the minor, see Fig. 1) were first observed² nearly ten years ago and studies of the properties of these nuclei^{3,4} have revealed many new phenomena and

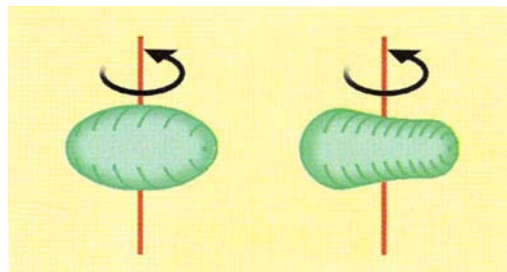


FIG. 1 Schematic of superdeformed (left) and hyperdeformed (right) nuclear shapes.

insights into the structure of nuclei. Calculations⁵ that predicted the existence of superdeformed shapes have been further developed to explain these features. These calculations also predict that at very high spins with angular momentum around $80\hbar$ (beyond the normal fission limit), the hyperdeformed shape (major:minor axis ratio = 3:1) becomes energetically favourable in nuclei with certain numbers of protons and neutrons. The inclusion of octupole (pear-shaped) correlations⁶ produces an asymmetry in the shape rather like a peanut for nuclei around atomic number $A=150$ (Fig. 1).

The initial signal of a hyperdeformed nucleus is a γ -ray sequence in which the energy difference between successive γ -ray transitions should be constant at about 30 keV, compared with a value of about 50 keV for superdeformed shapes. Scientists have scanned their data sets in superdeformed experiments but in only one case was there any indication of a positive signal, when a group from Chalk River Laboratories found tentative evidence⁷ for such a sequence in dysprosium-153. These nuclei were formed using a slightly different reaction, as a proton (in addition to the usual several neutrons) was emitted from the compound nucleus before the

γ -ray cascades. The experiment involved detecting the protons in a compact, efficient array, as well as the main γ -ray instrument.

In the past couple of years two very efficient γ -ray instruments, Gammasphere and Eurogam, have been built⁸. Gammasphere has been operated with the microball, a compact array of detectors which can efficiently detect and identify protons, α -particles and other light ions. It was this combination of instruments that produced the new evidence for hyperdeformation — two sequences of respectively 9 and 11 discrete γ -rays, each with energy separations close to 30 keV (as shown in Fig. 2).

Gadolinium-147 nuclei constitute only about 5 per cent of the products of the reaction of 230 MeV vanadium-51 with

a molybdenum-100 target. The proposed hyperdeformed bands are populated at the 0.3 per cent level in gadolinium-147 and therefore the combination of microball and Gammasphere is operating very close to its observational limit in observing these very weak γ -rays. The bands have the expected constant intensity over most of the sequence, decreasing at the highest transition energies. Although the average energy difference between γ -rays is 30 keV, individual values vary irregularly from 25 to 32 keV (with uncertainties of the order of 2–3 keV) and so, rather surprisingly, they are not as constant or smoothly varying as the separations in superdeformed bands.

No spin assignments can be unambiguously made to the hyperdeformed states as the γ -ray sequences have not been linked to known spin states, a situation that still prevails for superdeformed bands. But theoretical predictions are that the nucleus should behave like a rigid body, in which case the γ -ray energies indicate that the sequences would cover the extremely high spin range from about $65\hbar$ to about $90\hbar$. In comparison, the intensities of states in superdeformed bands drop rapidly above $60\hbar$ and none has been observed above $70\hbar$. This rapid decline is not caused by a lack of angular

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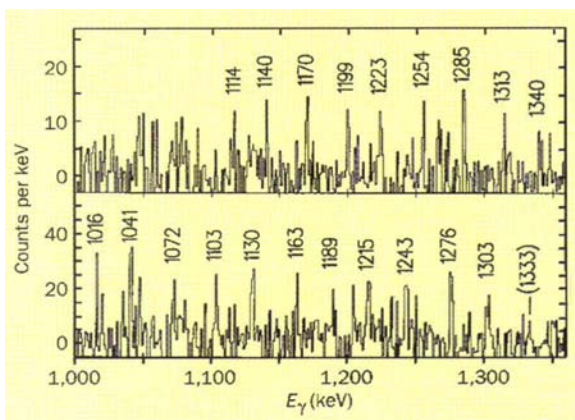


FIG. 2 Gamma-ray energy spectra of two sequences in gadolinium-147 showing the ~ 30 keV inter-peak spacing characteristic of the hyperdeformed shape. (After ref. 1.)

momentum in the reaction but by the dominance of the fission process at high angular momentum. If these new sequences are really associated with hyperdeformed shapes, then either the predicted spin values are much too high or the fission process must be drastically retarded for these extreme shapes. These are just some of the fascinating implications if hyperdeformation is the true explanation of the data.

The ultimate confirmation would be a measurement of the quadrupole moment which can be obtained from data on the lifetimes of the states. The normal method

of measurement — Doppler shift attenuation — would only provide a lower limit on the lifetimes, but this would show that the shape was at least superdeformed. In addition to this much longer experiment, angular correlation data are required to confirm that the transitions are of quadrupole and not dipole character. A third measurement is required to associate the bands unambiguously with gadolinium-147 and show that they are not observed until the maximum angular momentum in the reaction reaches a very high value of

well over $70\hbar$.

The next few months will see a flurry of activity, as groups in the United States and Europe attempt to answer these questions and show whether these sequences are indeed decays of hyperdeformed, peanut-shaped nuclei. Once again a new generation of powerful spectrometers is opening up new vistas in nuclear structure physics. □

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VIROLOGY

Evolving Darwinism

John F. Y. Brookfield

THERE have been three phases in the history of evolutionary genetics. The discipline was born in the neo-darwinian synthesis, in which mendelian genetics was reconciled with the ideas of Darwin. Then came the arrival of molecular techniques, which allowed the estimation of levels of polymorphism in proteins and base sequences, and showed that most DNA-sequence polymorphisms are neutral in their effects on fitness. Now, a third phase is emerging, perhaps to be called post-post-neo-Darwinism. The emphasis is once more on the small minority of base changes that are subject to positive selection¹, the selective effects of new mutations being the subject of especial attention. In this area, a new study of adaptive evolution in the RNA-based vesicular stomatitis virus, reported by Novella and co-workers in *Proceedings of the National Academy of Sciences*², is a classic.

Before the advent of molecular techniques, genetic polymorphisms were identifiable only when they resulted in easily observable phenotypic effects, likely to be

subject to natural selection. But when people began to look at DNA sequences, it became clear that the vast majority of base changes are neutral or nearly neutral in their effects³. Because the bulk properties of DNA evolution were dictated by this neutral majority, regularities stemming from neutrality, such as the independence of population size and evolutionary rate, led to expectations that rates of evolution would be relatively constant within lineages (hence the concept of molecular clocks). Furthermore, the expected lack of convergence in neutral changes allowed phylogeny to be estimated by parsimony, and gene flow and population movements to be investigated through the geographical structure of genetic variability.

In a more fundamental sense, however, the question of whether the majority of DNA sequence changes in evolution are selected or neutral is of little intrinsic interest. However small a proportion they form of total evolutionary changes, it is the needle of adaptive substitutions in the haystack of neutral changes which is

where the real evolutionary interest lies for biologists. What are the effects of such mutations? More specifically, what is the frequency spectrum of the selective effects of new mutations? Does this frequency spectrum remain constant in the face of previous adaptive substitutions? These questions are dauntingly difficult, and the best hopes for answers are likely to come from experiments with microorganisms.

Escherichia coli can be used to study adaptive changes in laboratory environments, in which populations are started from single clones, and so both the generation and the fixation of advantageous mutations occur during the course of the experiment⁴. Generally, a plateau is reached where the rate of increase in fitness drops almost to nothing. The RNA viruses evolve even more rapidly. They have high mutation rates per base, yet their small genomes mean that the overall mutation rate is insufficiently high to cross the 'error threshold'⁵ beyond which maintenance of the best adapted variant becomes impossible. High mutation rates allow exceptionally rapid evolution, in which major adaptive changes occur in short-term experiments.

Novella *et al.*² passed a genetically marked strain of vesicular stomatitis virus (VSV) repeatedly (up to fifty times) through either HeLa or BHK21 cells. The resulting viruses were made to compete against a standard strain that was isogenic with the starting strain except that it had lost the monoclonal-antibody-resistant mutation seen in the experimental strain. One prediction was abundantly confirmed, in that the increase in viral fitness was strongly dependent upon the population size. Mutations affecting fitness are more likely to be deleterious than advantageous, and in small populations, in which the power of selection is attenuated, evolutionary changes reflect this expected harmfulness. In large populations, deleterious mutations will merely produce a background selection reducing effective population size. Changes in fitness depend upon the numbers and selective advantages of advantageous mutations, and will be positive.

The really striking result, however, is that Novella *et al.* found that the increase in mean fitness was almost exactly exponential. This is surprising for two reasons. First, advantageous mutations required to increase fitness arise randomly, and this creates a variance with time in the rate of fitness increase. Second, the proportion of new mutations that are advantageous might be expected to drop as the viruses adapt to the environment of the cells.

How general might this observation be? The frequency spectrum of the selective effects of new mutations is irreducibly a consequence of all the biology of the organism, and its relationship with its

environment. So there is no reason for different species to be similar in their spectra. This problem may not be soluble through model systems, where experimentally tractable organisms are examined, and inferences drawn about other, more complex, ones. The regularity of fitness increase in VSV, however, suggests that there is some, previously unsuspected, biological reason why mean fitness increases regularly with time. Does this imply an underlying regularity in the distribution of advantageous effects of new mutations which may hold for many organisms? It is a tantalizing possibility, but seems unlikely.

In viruses the expected genetic diversity will be high. The introduction of variability depends on the product of population size and genome-wide mutation rate, while the loss of variation depends upon the rate of fixation, determined mainly by selection coefficients. The distribution of selection coefficients may be similar in RNA viruses to that in other evolving systems, but the mutation rate, even allowing for small genome size, will be

greater. Thus viral populations may not show the periods of no change in fitness that arise through populations waiting for the next advantageous mutation. The absence of a plateau in fitness seen in VSV also does not seem to be general.

Nevertheless, many types of RNA viruses are known, and to measure the evolutionary dynamics of adaptation in some of them should be relatively straightforward. It will be interesting to see if the properties of VSV are mirrored by those of other viruses. □

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QUASARS

A spectre in the spectrum

G. Mark Voit

ASTRONOMERS have known for decades that winds approaching 10 per cent of the speed of light emerge from many quasars. How quasars propel such rapid winds has remained unclear. On page 576 of this issue¹, Arav and collaborators present tantalizing evidence suggesting that these outflows attain their high velocities by tapping the flood of photons streaming from the quasar's central engine.

Huge luminosities and emission lines with widths of several thousand kilometres per second are the defining features of quasars. A substantial subset, about one in seven, also display broad absorption lines of highly ionized elements such as C³⁺ and N⁴⁺ that extend up to 30,000 km s⁻¹ to the blue side of the corresponding emission line². Somewhere outside the broad-line emitting region, thought to lie 10¹⁰ to 10¹³ km from the central continuum source, ionized plasma accelerates outwards prodigiously. This phenomenon is likely to be even more common than it appears. Detailed line-profile studies show that the absorbing gas subtends a relatively small fraction of the solid angle seen from the centre³. Most quasars probably have strong winds, detectable only in the 15 per cent whose random orientations happen to place absorbing material along our line of sight.

Radiation pressure would seem to be the obvious way to drive an outflow. The broad absorption lines clearly show that

photons emitted from the central source in our direction transfer a significant fraction of their momentum to the absorbing gas. Hot stars drive winds at 1,000–2,000 km s⁻¹ by such a process and produce absorption lines with qualitatively similar profiles. What prevents quasars from acting similarly?

Theorists attempting to model quasar winds must solve two problems: the outflow must absorb sufficient momentum, and it must not become too highly ionized while accelerating. Unlike hot stars, quasars copiously radiate X-rays that can strip all the electrons from the ions in the flow, drastically lowering its opacity and shutting off the acceleration. To reach high velocities, a wind pushed by photon pressure on absorption lines must either be shielded from X-rays or compressed so that its degree of ionization remains steady^{4,5}. These possibilities have recently received renewed attention^{6–8}. Although some aspects appear promising, the current models are still too simplistic and incomplete to account for the variety and richness of structure observed in quasar absorption-line profiles⁹. This lack of contact between theory and observation has made it difficult to prove that photons do accelerate quasar winds.

Hints of such a proof now appear in the spectra presented by Arav and collaborators¹. In a certain, carefully restricted, sample of broad-absorption-line quasars

seems to lurk a ghost that would arise only if radiation pressure propelled the quasar winds. The apparition is a local feature of enhanced flux in the C iv 1,550-Å absorption line at a velocity about 5,900 km s⁻¹ to the blue side of the line centre. This velocity shift happens to be equal to the velocity-space separation between the H I Lyman-α line at 1,216 Å and the 1,240-Å resonance line of N v. When the wind accelerates to about 5,900 km s⁻¹, Lyα photons from the quasar shift to about 1,240 Å in the wind's reference frame and are absorbed by N v. If the Lyα pressure on N v is a significant fraction of the total, acceleration of the wind through this velocity range is especially strong. The resulting paucity of gas at these velocities renders the wind more transparent, producing a small peak in the broad valley of the absorption profile.

In most quasars, the relative importance of Lyα pressure is too small to produce a distinct ghost¹⁰. The resonance transitions of many common ions belong to the ionizing portion of the ultraviolet continuum, which is typically quite strong in quasars. Recognizing that the pressure on all these lines would overwhelm the Lyα contribution, Arav *et al.* have identified a few quasars that are dim in the ionizing ultraviolet band, whose strength is inferred from the He II recombination line at 1,640 Å. They find that ghosts appear when the He II emission lines are weakest and the Lyα pressure is supposedly most important.

Assessing the weight to give to this result is tricky. Ghost-like features appear near the expected position in about 20 per cent of quasars with deep absorption features. By applying four criteria considered necessary, on theoretical grounds, for a ghost to appear, Arav *et al.* reduce the original sample of 72 quasars to a subsample of eight. Three of the eight host Lyα ghosts, and these three have the weakest He II emission lines. A fourth quasar from outside the original sample satisfies all the criteria, is weak in He II, and displays a ghost in its O vi 1,035-Å absorption line. So all four of the objects satisfying all the necessary criteria have Lyα ghosts. Arav *et*

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al. optimistically set the odds of this happening by chance at one in 10^3 . More pessimistic individuals might worry that some of their criteria, such as the critical He II line strength and the definition of a ghost-like feature, are somewhat subjective and have been selected with the data in hand. These pessimists, who know that many ghosts haunt the graveyard of alleged discoveries based on *a posteriori* statistics, will await a more systematic confirmation of

the anti-correlation between He II emission and ghost strength, perhaps with a larger sample, before believing in Ly α spirits. In the meantime, this news will add momentum to the ongoing theoretical investigations of photon-driven winds from quasars. □

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SIGNAL TRANSDUCTION

A target for PI(3) kinase

Julian Downward

STIMULATION of cells with serum or purified growth factors results in the triggering of multiple signal transduction pathways, cascades of information transfer that are initiated at the cell membrane and culminate in the nucleus, ultimately to control gene expression and cellular proliferation. The function of these signalling pathways is subverted in cancer cells, which receive instructions to grow when they should not. One of these signalling systems involves the lipid kinase phosphatidylinositol-3-OH kinase, or PI(3)K, which phosphorylates the hydroxyl group at position 3 on the inositol ring of phosphoinositides — a key activity that is switched on by a huge number of extracellular stimuli. Relatively little is known, however, about the mechanisms that transmit the signal beyond this point. Now a report from Burgering and Coffey on page 599 of this issue¹, together with a recently published paper in *Cell* from the groups of Tschlis and Kaplan², add significantly to this picture by placing a proto-oncogene product, the serine/threonine kinase known variously as protein kinase B (PKB), Akt and Rac, downstream of phosphatidylinositol-3-OH kinase.

Akt/PKB was found independently as a result of its homology with protein kinase C and protein kinase A by two groups: Coffey and Woodgett named it PKB³, while Hemmings's group used the name Rac⁴. At the same time, Tschlis and co-workers⁵ identified the same kinase as the product of the *v-akt* oncogene of the rodent acutely transforming retrovirus AKT8 (ref. 6). To avoid confusion with the Ras-related GTPase Rac, this kinase will be referred to here as Akt/PKB. The new results indicate that Akt/PKB is activated by growth factors operating through phosphatidylinositol-3-OH kinase^{1,2} (see figure), raising the intriguing possibility that Akt/PKB might bind directly to, and be stimulated by, 3-phosphorylated phosphoinositides.

Akt/PKB possesses an amino-terminal pleckstrin-homology (PH) domain: similar domains in other proteins have

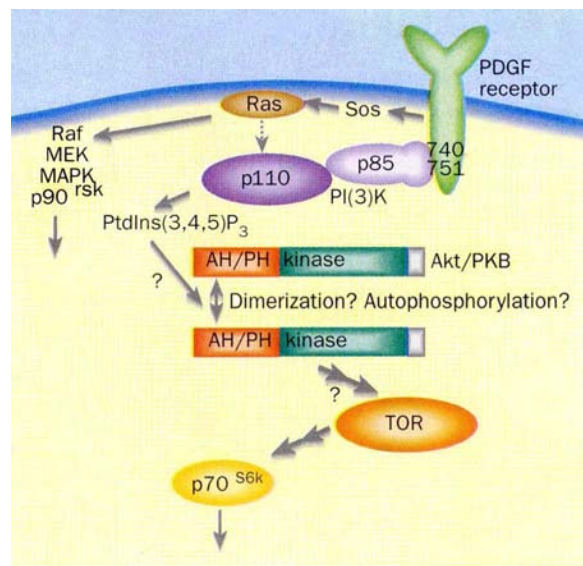
been implicated in interactions with phosphatidylinositol-4,5-bisphosphate, although not as yet with 3-phosphorylated phosphoinositides, and with other proteins, particularly heterotrimeric G-protein $\beta\gamma$ subunits. It has recently been shown that the first 147 amino acids of Akt/PKB, which include the PH domain, can mediate a stimulatory homodimerization of the kinase⁷. Point mutations in the PH domain also block the activation of Akt/PKB by growth factors².

It is possible that the products of phosphatidylinositol-3-OH kinase activity, such as phosphatidylinositol-3,4,5-trisphosphate, can promote dimerization of Akt/PKB and so activate it, possibly by inducing it to autophosphorylate. A similar synergy between phosphoinositide-protein and protein-protein interactions has recently been reported for the β -adrenergic-receptor kinase PH domain⁸. Indeed, Franke and colleagues² find that phosphatidylinositol-3-monophosphate can weakly stimulate the kinase activity of Akt/PKB, but as the level of this lipid is not increased following treatment with growth factors, the significance of this finding is not clear. It will be important to determine the effects of phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate, both of which are influenced by growth factors, on Akt/PKB activity *in vitro*.

Two other protein kinase activities may be regulated by phosphatidylinositol-3-OH kinase. One is the protein kinase C family, some members of which can be directly stimulated *in vitro*

by 3-phosphorylated phosphoinositides⁹. The other is p70^{S6k}, the ribosomal protein S6 kinase, which is activated in response to a wide variety of mitogens, but is inhibited by treatment of cells with supposedly specific inhibitors of phosphatidylinositol-3-OH kinase such as wortmannin¹⁰. The exact mechanism by which p70^{S6k} is regulated is still somewhat unclear and certainly involves several pathways. The fact that Akt/PKB activation involves phosphatidylinositol-3-OH kinase raises the possibility that it also contributes to control of p70^{S6k}.

Burgering and Coffey¹ explore this possibility: they show that activated mutants of Akt/PKB stimulate p70^{S6k} in cotransfection assays. Although this points to a role for Akt/PKB upstream of p70^{S6k}, to be certain it would be necessary to demonstrate that a dominant-negative mutant can block p70^{S6k} activation, because effects seen in cotransfection assays can often be due to autocrine secretion of growth factors. A further complication is the fact that a tyrosine-to-phenylalanine mutation at position 740 of the platelet-derived growth factor (PDGF) receptor, which causes a greatly reduced activation of phosphatidylinositol-3-OH kinase and Akt/PKB, does not affect the activation of p70^{S6k} (ref. 11). The exact role of Akt/PKB in p70^{S6k} regulation is still unclear, but should



A possible scheme for regulation of Akt/PKB by phosphatidylinositol-3-OH kinase (PI(3)K). p85 and p110 are the regulatory and catalytic subunits of phosphatidylinositol-3-OH kinase. The pleckstrin-homology (PH) domain of Akt/PKB encompasses amino acids 1–106, and the larger Akt-homology (AH) domain spans amino acids 1–147. Lipid products of phosphatidylinositol-3-OH kinase, such as phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P₃), may bind to the amino-terminal regulatory AH or PH domains of Akt/PKB and promote their dimerization and/or kinase-domain autophosphorylation, resulting in activation of the protein kinase activity. Possible downstream targets of Akt/PKB could include components of the p70^{S6k} pathway¹⁰, such as TOR, a rapamycin-sensitive kinase first characterized in yeast.

provide an impetus for future research.

One apparent discrepancy between the two reports is over the role of Ras. Franke *et al.*² show that dominant-negative mutant Ras inhibits Akt/PKB activation by PDGF, while Burgering and Coffey¹ find only slight inhibition. This could be due to cell-type variation or to methodological differences. Both groups agree that stimulation of Ras is not sufficient to activate Akt/PKB. As Ras interacts directly through its effector site with the catalytic p110 subunit of phosphatidylinositol-3-OH kinase and is involved in stimulating this lipid kinase activity in intact cells^{12,13}, it might be expected that Ras function would be necessary for Akt/PKB activation by growth factors. But the ability of dominant-negative Ras to inhibit Akt/PKB activation would depend both on the levels of expression of each transfected protein and on the amount of redundancy in the signalling pathways operating between the growth factor receptor and Akt/PKB, which can depend on cell type, as in the case of MAP kinase activation¹⁴. Given that chemical inhibitors of phosphatidylinositol-3-OH kinase do not inhibit activation of Ras by growth factors¹⁵, a model in which phosphatidylinositol-3-OH kinase plays a significant role upstream, rather than downstream, of Ras¹⁶ seems unlikely. The exact role of Ras in Akt/PKB regulation still needs clarification.

Although details of the connection between phosphatidylinositol-3-OH kinase and Akt/PKB still need to be filled in, the new developments raise the exciting possibility that the serine/threonine protein kinase Akt/PKB could be one of the long-sought targets of 3-phosphorylated phosphoinositides. The challenge is now to determine the nature of the cellular substrates of Akt/PKB and how they contribute to the control of cell proliferation and to malignant transformation. □

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Wrestling with restless calderas and fighting floods of lava

Ken Hon and John Pallister

GIGANTIC volcanic eruptions capable of devastating thousands of square kilometres and potentially plunging the Earth into volcanic winter simultaneously capture and stretch the imaginations of scientists studying them. Eruptions of this scale (10^2 – 10^3 km³ in volume) are infrequent, but, curiously enough, include both silicic ash erupted explosively from large calderas and huge flood basalt flows erupted more passively from extensive fissure systems. A desire to explore the common ground between eruptive processes, cli-

mulations that took place over longer periods of time. In this context, smaller historic eruptions, for example at Hawaii and Iceland or at Mount Pinatubo in the Philippines, can be scaled up and used as analogues for giant prehistoric flood basalt flows and pyroclastic flow deposits.

Pyroclastic flow deposits are reinterpreted (P. Kokelaar and M. Branny, Univ. Liverpool) as the progressive aggradation from particle-laden ash clouds, rather than representing the bodies of dense avalanches. In a similar

way, thick basalt flows are reinterpreted (K. H., US Geological Survey; S. Self, Univ. Hawaii) as initially thin sheets of lava that spread over flat areas and were later inflated by a slow-moving core of molten lava. Continuously growing crust not only retains pressure within the molten core but also insulates it, allowing the lava to travel great distances with little loss of heat. Large basaltic lava flows (over 200–400 km long) observed on gently sloping regions of other planets are relatively narrow, a feature more consistent with formation as sequentially emplaced inflated lobes than with rapid floods of lava (R. Lopes-Gautier, Jet Propulsion

Shuttle image of the eruption column and plume from Rabaul volcano, Papua New Guinea, taken in September 1994.

mate effects, precursory activity, potential hazards and magmatic plumbing of these seemingly disparate types of eruptions brought together researchers for a recent symposium and related field workshop*.

There emerged a remarkably parallel, yet independent, rethinking of emplacement models for large (10–100 m thick) pyroclastic flow deposits and flood basalt flows, one that calls for progressive rather than *en masse* deposition. The implications for eruption rates, global climate and volcanic hazards are profound, as is the effect on our understanding of the role of catastrophism in Earth history. Eruptive deposits that were previously thought to represent near-instantaneous accumulations from huge floods of fast-moving lava or from single dense avalanches of ash may be better viewed as incremental accu-

Lab.; J. Zimbelman, Smithsonian Inst.).

Ironically, the longer emplacement times inferred from the new models, especially for flood basalts (years to decades), may intensify their effects on global climate through long-term atmospheric loading (Self). For example, the 1783–84 Laki basaltic eruption in Iceland produced extreme cooling over northern Europe and resulted in widespread famine. The Laki eruption, although large by historic standards (15 km³ of lava), is two orders of magnitude smaller than many ancient flood basalts. For example, the Columbia River Basalt group consists of about 300 lava flows that were erupted between 17 and 6 million years ago and cover an area of about 164,000 km² in the northwestern region of the United States (S. Reidel, Westinghouse Hanford Co., Washington; T. Tolan, Portland State Univ.). Many of these lava flows individually exceeded 1,000 km³ in volume and travelled hundreds of kilometres from their vents. Estimates of sulphur dioxide output for

*XXI IUGG Symposium on Evolution of Large Volcanic Systems and Restless Calderas; Workshop on Columbia River Basalt and Yellowstone Volcanic Field, Boulder, Colorado, USA, 25 June–4 July 1995.

such a Columbia River Basalt eruption could be of the order of 1,000 megatons per year, which would produce atmospheric conditions similar to those modelled for nuclear winter.

Most flood basalt provinces are associated with continental break-up and, fortunately, don't pose an immediate hazard as they occur relatively rarely (once in 10^7 – 10^8 years) in the geological record. However, an association with periods of mass extinction suggests that they may have played a role in past climate change. Large (10^2 – 10^3 km³) silicic, caldera-forming eruptions may also play a part in shifting climate during unstable periods, a role suggested for the eruption of the Toba caldera in Indonesia 74,000 years ago (Self). The recurrence interval for such large caldera-forming events is around 10^5 – 10^6 years, but smaller caldera-forming eruptions (10–100 km³) such as Rabaul (Papua New Guinea), Campi Flegrei (Italy) and Lake Taupo (New Zealand) occur every 10^3 – 10^4 years. These eruptions are less likely to produce long-lasting climate change, but pose much more serious threats to society.

Because even the smaller caldera-forming eruptions are infrequent and the resulting volcanic structures are complex, forecasting events at 'restless' calderas known to be seismically and geodetically active is difficult (D. Hill, US Geological Survey). Data on precursory activity remain sparse even from the most restless calderas and non-eruptive seismic crises at calderas can be considerably more intense than precursory seismicity typical of actual eruptions at some smaller volcanoes. Another concern is the current inability to distinguish reliably between eruptive and intrusive ('failed eruptive') events in order to avoid issuing what appear to be 'false' forecasts to the public. The very short warning period (27 hours) that preceded the 1994 eruptions from Rabaul caldera (C. McKee, Rabaul Volcano Observatory) demonstrated just how unpredictable caldera systems can be. Fortunately, a decade of intermittent unrest and excellent public awareness at Rabaul led to a timely evacuation of about 100,000 people with only five fatalities. An evacuation on a similar scale seems to have occurred before the Bronze Age eruption of Santorini caldera (G. Heiken, Los Alamos Lab.), suggesting that precursory activity was sufficient in this case to convince even the most stubborn to abandon the island.

One can only hope for such success if an eruption were to result from current episodes of unrest reported from the Campi Flegrei caldera in the Bay of Naples (L. Civetta, G. De Natale, G. Orsi, Osservatorio Vesuviano). This is arguably the world's most dangerous volcanic field, with more than 1 million people in the region sandwiched between the caldera

and Vesuvius. Large eruptions 36,000 and 12,000 years ago formed nested calderas, and recent seismic and geodetic activity outlines a smaller area within the caldera structure that is moving in response to changes in a shallow magma body and/or changes in shallow aquifers. The Yellowstone caldera of Wyoming is currently deforming over a much broader area (C. Meertens, Univ. Utah), but it is also difficult to assess whether this is the result of changes in the deep hydrothermal system or interplay between magmatic and regional tectonic systems. Finally, at calderas with long histories of both rhyolitic and basaltic volcanism, like Yellowstone and Long Valley, California, "We can't be sure if a forecast event will be a basaltic tourist attraction or a hazardous, explosive rhyolitic eruption" (Hill).

Generation, evolution and storage of the huge amounts of magma required to fuel large caldera-forming and flood basalt eruptions remains a topic of great interest. Estimated sizes of the magma reservoirs for these eruptions (10^3 – 10^4 km³) range from twice to 10 times the volumes of individual eruptions. Flood basalts are not direct mantle melts; they have undergone considerable differentiation, and may have assimilated some of the surrounding lower crustal rocks in magma chambers that are thought to be

localized near the boundary between the crust and mantle (R. Carlson, Carnegie Inst.). During late stages, silicic volcanics may be produced in the same areas as the flood basalts, but these differ from most caldera-related silicic volcanics in having very high eruptive temperatures (over 1,000 °C) and compositions indicative of melted anhydrous lower crust (A. Duncan, Univ. Cape Town). The fate of basaltic magma that ponds higher in the crust may be to trigger extensive melting of surrounding hydrous crustal rocks, the melts of which probably do feed subcaldera magma chambers and granitic batholiths (J. Quick, US Geological Survey). The transfer of thermal energy to these easily melted mid-crustal wallrocks causes the basaltic magma to produce large accumulations of crystals, which deform in a viscous manner and flow away from the intrusive axis. This transformation would produce rocks that appear much like the highly deformed middle crust they intruded and may provide an answer to one of the most perplexing questions in volcanology: what has happened to all the basalt that should be beneath frozen silicic magma chambers? □

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PALAEONTOLOGY

Ecological apes and ancestors

Peter Andrews

THE competition for the oldest hominine seems to be hotting up. White and colleagues recently described a collection of specimens from Pliocene deposits at Aramis in Ethiopia that they named a new genus and species of hominine, *Ardipithecus ramidus*^{1,2}. In this issue, on page 565, Meave Leakey and others describe another new species of hominine from sites at Kanapoi and Allia Bay in northern Kenya³. Both these sites are slightly younger than the Aramis date of 4.5 to 4.3 Myr (million years ago)⁴. At Kanapoi the hominines are found at two levels, one well dated at 4.1 Myr and the other from younger deposits older than 3.5 Myr, whereas the deposits at Allia Bay are dated to around 3.9 Myr³. (Note that hominine is used in preference to hominid as the family Hominidae is now usually taken to include the African apes as well as humans, Homininae being restricted to members of the human clade.)

Most of the fossils from Kanapoi come from the lower level, and there are some surprises there. The type specimen is a mandible with symphysis and complete dentition. It is distinguished from the slightly younger *Australopithecus afar-*

ensis by a suite of characters, clearly justifying the species distinction, but comparisons can also be made with fossil apes from the middle to late Miocene, and when this is done a number of interesting similarities emerge. For example, the African kenyanthropines combine the features of a strongly posteriorly defined symphysis, with long postincisive plane, seen also in *Griphopithecus* from Turkey⁵. Also present in both fossil apes are shallow palates, largish upper and lower canines with vertical roots, asymmetrical premolar P₃s with a mesial beak in *Griphopithecus*, upper molars with trigons wider than talons and thick enamel. All these are described as features of the Kanapoi hominid³.

In striking contrast to the dental similarities of the Kanapoi specimens with fossil apes, the postcrania tell a different story. They come from the upper level at Kanapoi, in contrast to the type specimen which comes from the lower level. Included here is the distal humerus which was found 30 years ago⁶ and which has been placed in stratigraphic context by examination of old photographs of the site (M. Leakey, personal communication). This fossil

Social conscience

IMPRISONING criminals is an expensive way of preventing crime. A cheaper scheme is to fit each offender with a radio 'tag' to report suspicious movements. So far, this has not worked very well.

The old-fashioned way of preventing crime was to equip all citizens with an internal inhibitor called a 'conscience'. It was routinely established at an early age. You waited till the child did something you didn't like, and then you shouted at it or hit it. At all other times you were kind to it. This simple procedure worked well, and is still used with great success on domestic animals. It depends on standard conditioning theory, which requires the punishment to follow the crime so swiftly that the subject instinctively learns to associate the two.

Modern enlightenment has abandoned such barbarity. Punishment can now be meted out, if at all, only after slow and complex routines of authorization — by which time the offender has long since forgotten its connection with the crime. Unwarned and undeterred, he goes on to commit more serious crimes. Daedalus is now devising an electronic tag which applies its own instant conditioning.

His 'smart tag' records a lot more about its wearer than his mere position. Sensors against his skin register his temperature, blood pressure and pulse rate; a microphone records his speech and the sounds around him; a set of accelerometers senses his pattern of movement. After a little experience, the tag's neural-net processor can instantly deduce what he is up to. The physiological and kinetic signatures of (say) burglary, or mugging, or breaking into a car, or spraying graffiti, are highly individual and easily recognized. When it spots such activity, the smart tag ceases to be a mere passive observer of its wearer. It transmits a signal to the police; more to the point, it also emits a startling high-pitched scream and delivers an instant, painful electric shock.

Even the dimmest criminal will rapidly learn, by direct association, that crime does not pay. Daedalus's 'electronic conscience', with its minor but instant and certain punishment, will prevent crime far more efficiently than the law, with its drastic, expensive, but highly uncertain and always long-delayed retribution. Even better, the tag's steady conditioning will gradually establish an old-style conscience in the wearer. In due course he will be so well conditioned against crime that his electronic conscience can be removed; his newly acquired internal one will do the job. Only sociopaths and hopeless recidivists, incapable of reliable learning, will need to wear it permanently. David Jones

bone was recognized early on to be more advanced than australopithecines and was found to group morphologically with *Homo*⁷, a surprising conclusion because the Kanapoi deposits are not only much older than any deposits containing fossils of early *Homo* but are also older than many australopithecine sites. The present discoveries reinforce that conclusion, however, for a partial tibia found at the same level as the humerus also shows some *Homo*-like characters³, particularly in its overall length (estimated because the midshaft is missing).

Body-size estimates based on the sizes of the two ends of the tibia indicate a range

will be needed to support this allocation.

Comparisons of Pliocene hominines are generally made with other, later, hominine species. At this early stage of human evolution, however, it is to be expected that there will be many similarities with fossil apes, and whether one works forward from the apes or back from humans, it is important that, like the channel tunnel, the two approaches should meet in the middle. Rather than questioning which hominid lineage the Kanapoi species may be on, therefore, I would like to raise the question of how it differs from fossil apes. Could it, for instance, belong to a bipedal ape not on the hominine lineage? Based on the association of the *Homo*-like postcrania, could it belong to a hominine lineage separate from the australopithecines but more directly intermediate between fossil apes and *Homo*? If the upper and lower Kanapoi specimens prove to be taxonomically distinct, there is even the possibility that one group may be related to humans and the other to apes.

In addition to these phylogenetic questions, there is the issue of the environment and life history of the Pliocene hominines. There are increasing indications that some of the early hominines are associated with closed woodland/forest environments, and it has also been shown that, although they had bipedal adaptations, hominines like *A. afarensis* retained some adaptations to an arboreal way of life⁸. The recent description of four articulating footbones from 3–3.5 Myr deposits in the South African cave site of Sterkfontein supports this for, although primarily adapted for bipedalism, the divergent big toe indicates some degree of prehensile grasping as in apes⁹. Developmental patterns were also more ape-like than human¹⁰. Whether they were phylogenetically hominines or not, it seems to me that ecologically they may still be considered as apes. □

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Aramis	Thin-enamelled hominine with australopithecine-like postcrania
Lower Kanapoi	Thick-enamelled hominine with jaws like fossil apes, unknown postcrania
Upper Kanapoi and Allia Bay	Hominine with <i>Homo</i> -like postcrania
Laetoli	Thick-enamelled hominine with possible <i>Homo</i> affinities and bipedal gait
Hadar	Thick-enamelled hominine with australopithecine-like postcrania

of 46–55 kg for this individual, larger than the contemporaneous hominine *Australopithecus afarensis*. The new species is larger also than *Ardipithecus ramidus*, which retains chimpanzee-like postcrania¹. The latter is also distinguished by its thinner tooth enamel, which may be ancestral or derived depending on how thickness polarity is determined. Several other differences are also noted in the species diagnosis, probably justifying the distinction from *A. ramidus*, but there is only limited overlap in body parts preserved in common in the two hominids. Unfortunately, there are no postcrania from the lower level at Kanapoi to compare either with *Ardipithecus* or with upper level Kanapoi. A summary of the apparent morphologies of Pliocene hominines is shown in the table.

There are several issues that arise from these new discoveries from Kanapoi and Allia Bay. One concerns the linking of the upper Kanapoi postcranial material with the dental specimens which come from the lower Kanapoi deposits. If the dating is correct, and if there is just one species represented at Kanapoi, it results in the remarkable combination of advanced *Homo*-like features of the postcrania (that is, the humerus and tibia from the later deposits) with primitive jaws and teeth similar to those of Miocene apes. This combination is at odds with the more usual australopithecine pattern, and as the mandible from the upper level has no teeth in place, and there are no postcrania from the lower level, additional material

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Why is dieting so difficult?

Sir — Dieting for cosmetic or medical reasons is common in developed countries, but is surprisingly ineffective as a means of achieving sustained weight loss¹. We have previously established that moderate dieting alters the activity of 5-hydroxy-tryptamine (5-HT), a brain neurotransmitter involved in regulating appetite and food intake². Specific membrane receptors for 5-HT exist in many subtypes, possessing diverse functional roles. Tecott *et al.*³ have shown that mutant mice lacking 5-HT_{2C} receptors become obese through abnormal control of feeding behaviour, in agreement with other animal experimental data suggesting that 5-HT_{2C} receptors in the hypothalamus are important for controlling food intake.

We studied the effect of moderate dieting in 12 healthy women (age 20–39 yr) on the prolactin responses to the 5-HT_{2C} receptor agonist *m*-chlorophenylpiperazine (mCPP), a measure of the sensitivity of hypothalamic 5-HT_{2C} receptors⁴. Subjects received a double-blind, randomized, placebo-controlled challenge with mCPP (0.25 mg per kg, orally) in the early follicular phase of their menstrual cycle, and one week later started a 1,000-kcal daily diet. The mCPP/placebo challenges were repeated 3 weeks later at the same stage of the menstrual cycle, by which time subjects had achieved a mean weight loss of 3.1 ± 0.2 kg. Following dieting, the effectiveness of mCPP in increasing plasma prolactin levels was significantly enhanced, but plasma concentrations of mCPP were unaltered (data not shown). Consistent with our previous work², dieting significantly lowered plasma concentrations of tryptophan, the amino-acid precursor of 5-HT, which resulted in a

decreased plasma ratio of tryptophan to branch-chain amino acids (leucine, isoleucine and valine; 0.032 ± 0.003 as opposed to 0.026 ± 0.001 ; $P = 0.014$).

A decrease in the ratio of tryptophan to branch-chain amino acids would be expected to lower the availability of tryptophan to the brain and reduce brain 5-HT synthesis². Thus dieting lowers plasma tryptophan, thereby decreasing brain 5-HT and producing the compensatory upregulation in the responsiveness of 5-HT_{2C} receptors which we have detected. Dieting therefore seems to decrease neurotransmission at hypothalamic 5-HT_{2C} receptors by reducing the availability of their neurotransmitters. How might this affect dieting behaviour?

Just as the absence of hypothalamic 5-HT_{2C} receptors causes mice to overeat, medications that antagonize 5-HT_{2C} receptors, such as the antidepressant mianserin and the antipsychotic agent clozapine, cause troublesome weight gain in clinical use. Thus, during dieting subjects are likely to experience urges to overeat which will compromise efforts at continued food restriction. Indeed, diet-

ing is well recognized as causing disordered eating patterns which may evolve into clinical eating disorders such as bulimia nervosa⁵, a condition characterized by uncontrolled episodes of overeating. Humans have evolved powerful adaptive mechanisms for maintaining food intake. Trying to overcome these by voluntary food restriction is not only difficult but may be counterproductive in some individuals. On the other hand, appropriate manipulation of 5-HT_{2C} receptor function may benefit those in whom weight loss is indicated for clinical reasons.

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Differential abortion in the yucca

Sir — Pellmyr and Huth¹ showed that the yucca *Yucca filamentosa* can prevent over-exploitation by the yucca moth *Tegeticula yuccasella*, a pollinator/seed predator, through differential abortion of infested fruits. Our work on *Yucca whipplei typica* and *Tegeticula maculata* confirms these findings, but suggests that over-exploitation may be a minor selective pressure maintaining differential abortion.

At maturity, *Y. w. typica* produces a single inflorescence of 500–1,500 flowers². *Tegeticula* females, the plant's sole pollinator, actively place pollen on yucca stigmas, usually after laying one or a few eggs in the flower's ovary. On average, a resulting larva consumes 7.7 of the 174 available seeds³. The consumed seeds are viewed as the plant's fitness cost to maintain the mutualism.

Typically, 50% of the initiated fruits are aborted early in development, apparently due to resource limitations^{3,4}. Given the limited number of fruits that can be matured, retention of higher-quality (less infested) fruit should increase reproductive success and thus mitigate the cost of mutualism by shifting costs back onto the pollinator.

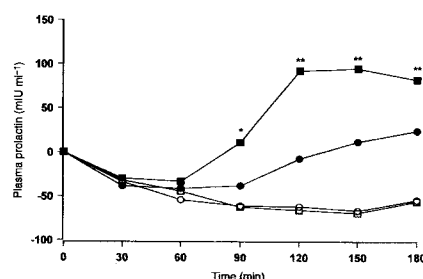
We compared abortion rates of fruits that received no oviposition (hand pollinated) with those exposed to oviposition (open pollinated) on each of seven plants.

Retention of uninfested fruits was higher on six of the plants (no difference on the seventh; $P < 0.02$, sign test). In another study, we counted eggs in 26 pairs of aborting and non-aborting fruits which occupied adjacent positions on racemes. Aborting fruits in the pairs had significantly more eggs (see figure).

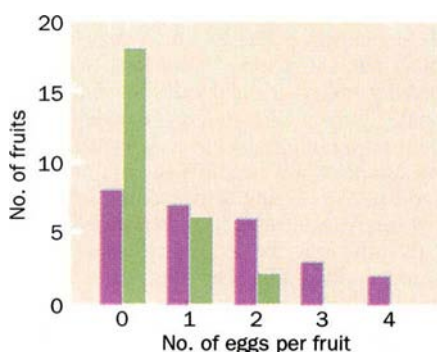
Two questions arise. First, is differential abortion a special mechanism for coping with *Tegeticula*, or a general adaptation? Second, how effectively does differential abortion reduce the costs of mutualism?

Differential abortion is a viable strategy for *Y. w. typica* because of its apparent overproduction of flowers. Pollen export increases with inflorescence size more strongly than seed set, which indicates that the 'extra' flowers contribute to plant reproductive success through male function³. This makes the female organs in many of the flowers expendable, and gives the plant the opportunity to selectively mature those that will develop into the best fruits. Other *Yucca* spp. abort fruits with low seed set¹, and we found frequent abortion of fruits resulting from self-pollination (the plant is self-compatible)³. Thus, as with other species⁵, differential abortion is a general adaptation in *Y. w. typica*.

Cost reduction achieved by selectively aborting infested fruits is small. Given observed abortion rates, numbers of eggs



Mean plasma prolactin (measured as change from baseline) in 12 female subjects before (circles) and after (squares) double-blind oral challenge with mCPP (0.25 mg per kg; solid symbols) or placebo (at time 0; open symbols). Subjects were tested on two occasions before (pre) and at the end (post) of a 3-week, 1,000-kcal diet. Analysis of variance showed a significant interaction of mCPP, diet and time ($F = 5.73$; d.f. = 6,66; $P < 0.0001$); *, $P < 0.05$; **, $P < 0.01$ (Fisher's test of least significant difference).



Histograms comparing the numbers of *T. maculata* eggs contained in 26 pairs of aborting (pink bars) and maturing (green bars) fruits. Abort fruits contained more eggs (Wilcoxon's signed-ranks test, $P < 0.001$). Fruits were collected from 26 plants. Fruits within a pair were located directly opposite each other on the same branch. Pairs in which one was aborting and one was not were collected, then dissected to determine the number of moth eggs. The proximity of the fruits on the branch indicates that they were produced and exposed to oviposition at the same time.

per flower, and number of seeds destroyed per larva, we estimate that with random abortion, *Y. w. typica* would mature 167 seeds per fruit, whereas differential abortion resulted in an average of 174 seeds — a 4% increase. By contrast, the impact of differential abortion on moth reproductive success is severe. On average, 0.83 eggs were deposited per fertilized fruit. Of these, 0.40 would survive if abortion were random, but only 0.19 actually survived under differential abortion — a decrease of more than 50%. Thus, the plant shifts a small amount of its cost in this mutualism back onto the pollinator in the form of egg mortality. Although the moth may mitigate this cost shift by depositing fewer eggs per flower, this mitigation must be balanced against the added expense of visiting more flowers to deposit the full complement of eggs.

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PELLMYR AND HUTH REPLY — We disagree with Richter and Weis's suggestion that over exploitation cannot explain the maintenance of differential abortion, on the notion that random abortion would cause only a slight decrease in mean number of intact ripe seeds. First, intact seed gain attributable to selective abortion was 9% in our original data¹, a fitness difference that (if fixed) would translate into considerable selection for

differential abortion. The difference in terms of eaten seeds is likely to be a function of pollinator density, rather than a fixed number. A given plant will be able to retain a certain number of flowers to ripe fruit, so that in years of high pollinator density it can retain a subset of flowers with far lower moth density than the average flower. With the Poisson-like egg distribution found in *Yucca filamentosa*, the selection differential will be positively correlated with pollinator density. *Tegeticula* density is highly variable between years and sites^{6,7}, so long-term data for pollinator density variation are needed to obtain a robust measure of variation in selection differential between random and selective fruit abortion in yuccas.

Richter and Weis's conclusion that selective abortion is a "general adaptation" agrees with our conclusion that it is a symplesiomorphy within the Agavaceae, and thus a preadaptation within the yuccas. In fact, it was the basis for our proposed model as to why selection could act on colonizing yucca moths to provide high-quality pollination. Our present experiments show strong positive effects on fruit retention of increased pollen load, outcrossing and genotype diversity in *Y. filamentosa*; this effect extends beyond pollen loads needed for complete seed set by a factor of at least ten, confirming the inferred positive effect of multiple moth pollinations suggested by our original dataset.

Why, then, do flowers with many moth pollinations suffer increased risk of abortion? The rapid death of ovules at the point of egg insertion could be a plausible proximal cause as to why flowers with many eggs fail in competition (despite high pollen loads), because they constitute a lesser sink relative to simultaneously developing fruit with more developing ovules. In this scheme, the moths carry the seed of their own demise in the form of an egg-linked factor triggering ovule death — perhaps a preadaptation in a lineage of moths whose more basal taxa oviposit into the flowers of host species without abortion of pollinated flowers^{8,9}.

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Age of early hominids

SIR — We read the announcement of the discovery and naming of *Australopithecus ramidus*¹ or *Ardipithecus ramidus*² by White *et al.* with great interest, but take issue with its age as reported by WoldeGabriel *et al.*³ and especially their interpretation of the results from their paleomagnetic sampling.

As noted by WoldeGabriel *et al.*³, the geological section at Aramis has several volcanic tuffs present throughout its exposures, but only the Gāala Vitric Tuff Complex which is found at a stratigraphic level below the hominid fossils has been shown to be datable. This tuff, although contaminated, shows one feldspar population having a mean age of 4.387 ± 0.031 Myr which is taken as the maximum age for the fossil hominids³. Other tuffs found immediately above the hominid fossil localities were shown to be contaminated and undatable³.

In an attempt to further constrain the age of the fossils, WoldeGabriel *et al.*³ sampled for palaeomagnetic reversal stratigraphy and collected two samples, one of which yielded uninterpretable results. The second palaeomagnetic sample is reported to be of reversed polarity and was collected from a level slightly below the undated Daam-Aatu Basaltic Tuff, both of which are from within the stratigraphic interval of the fossil localities. Although WoldeGabriel *et al.*³ argue that this single reversed sample constrains the *A. ramidus* material to subchron C3n.1r, this is not the case. Until other older and dated tuffs such as the Sibabi Marker Tuff³ can be correlated into the section at Aramis either physically or by chemical composition, or other tuffs at Aramis are dated, the only clearly available capping age for the fossils is that of 3.89 ± 0.02 Myr for tuff VT-1 (= Moiti Tuff)^{3,4}. The age range for the fossils should be given as 3.89–4.39 Myr, and the reversely magnetized rock sample reported could be from either the younger reversed interval of chron C2Ar (3.58–4.18 Myr) or the older reversed interval of subchron C3n.1r (4.29–4.48 Myr)^{5,6}.

Although future work at Aramis may provide more convincing support for an age assignment of *A. ramidus* to subchron C3n.1r, this determination will require either a clear identification of C3n.1n in the sediments that overlie the fossils, or an age determination of greater than 4.18 Myr for one of the tuffs found above the site of the single palaeomagnetic sample reported from Aramis. Until then, the age of *A. ramidus* should be reported as ranging between 3.89 and 4.39 Myr, rather than as "... around 4.4 million years of age" (ref. 1, p. 306; ref. 3, p. 330).

Interestingly, this age range shows near temporal overlap with a variety of specimens attributed to or suggested to show

Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and five references.

affinities with *A. afarensis* from Belohdelie, Ethiopia⁴, Allia Bay⁷ and Tabarin⁸, Kenya, and Fejej, Ethiopia^{9,10}, which suggests the likelihood of several contemporary species. It thus appears that the phylogeny of hominids, like that of many other mammalian groups is very bushy at its base

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WOLDEGABRIEL ET AL. REPLY — Our first published chronological assessment was made on the basis of stratigraphy, structural relationships, biochronology, $^{40}\text{Ar}/^{39}\text{Ar}$ dating and palaeomagnetic data³. Regional dip, coupled with stratigraphic and structural coherence, indicated that the Aramis strata were deposited well below the VT-1/Moiti tuff (3.89 ± 0.02 Myr). Biochronological comparisons with fauna from Maka/Belohdelie provided the first clue that the Aramis hominids were older than those from east of the modern Awash River.

These relationships were confirmed by $^{40}\text{Ar}/^{39}\text{Ar}$ dating combined with palaeomagnetic data. The palaeomagnetic sampling was not an attempt at "reversal stratigraphy" (an absurdity if based on two samples), but rather at testing for consistency with the $^{40}\text{Ar}/^{39}\text{Ar}$ results. We reported initial dating results of 4.387 ± 0.031 Myr for the Gāala Vitric Tuff Complex (GATC), providing a maximum age for the *A. ramidus* fossils³. As we stated, the palaeomagnetic data are consistent with the *A. ramidus* fossils being between 4.29 and 4.48 Myr old, based on the astronomically calibrated geomagnetic polarity reversal timescale¹¹. This likelihood seems to us far stronger than the younger age that Kappelman and Fleagle suggest.

Kappelman and Fleagle's conjecture would require that at least 210 kyr (from the GATC to above the top of the Cochiti subchron) be compressed into a thin stratigraphic section of less than 4 m without the trace of an unconformity. During this extended period the Aramis landscape in

this tectonically unstable area is required to have remained virtually featureless. This is because the superposed Daam Aatu Basaltic Tuff (DABT) maintains a uniform thickness across 4 km along strike of modern outcrop, indicating no subjacent topography. Furthermore, Kappelman and Fleagle's scenario implies that the entire thick succession of sediments (>121 m) above the DABT and below the VT-1/Moiti was then deposited within the next 290 kyr (from <4.18 Myr, the top of the Cochiti subchron, to 3.89 Myr, the age of VT-1/Moiti). There is no stratigraphic evidence for such an abrupt and profound increase in sediment accumulation rate (from <2 to >41 cm kyr⁻¹).

Due to the paucity of cognate feldspar phenocrysts in distal tuffs overlying the hominid fossils³, we have begun $^{40}\text{Ar}/^{39}\text{Ar}$ dating of fresh juvenile glass lapilli from the DABT and another, similar basaltic tuff (the Kullunta Tuff) located 63 m above it. Incremental laser-heating analyses of the DABT from two different locations yield plateau ages of 4.390 ± 0.068 and 4.384 ± 0.086 Myr, with a weighted mean of 4.388 ± 0.053 Myr, indistinguishable from our date of 4.387 ± 0.031 Myr for the

GATC 4 m below it. The stratigraphically younger Kullunta Tuff yielded a plateau age of 4.289 ± 0.055 Myr. The results are in direct opposition to Kappelman and Fleagle's hypothesis that the age of *Aridipithecus ramidus* was overestimated

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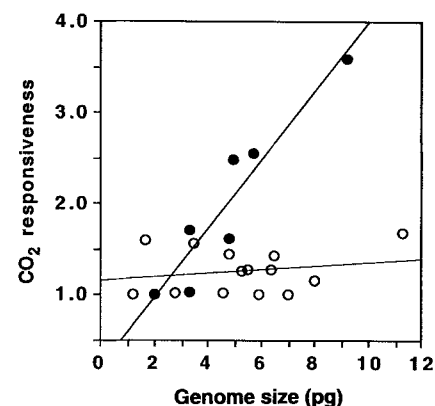
Genome size and high CO₂

SIR — Genome size, as measured by the content of nuclear DNA per cell, varies over 2,500-fold among species of angiosperm plants¹. As genome size is positively correlated with cell size and with the duration of cell cycle², it may have direct effects on the evolutionary strategy³, life history, phenology and distribution of species^{4,5}. Plant species with large genomes have longer generation times and can start growth at colder temperatures^{2,5}. Within species, smaller genomes are frequently associated with stressful or short-duration environments, whereas more beneficial conditions or cultivation lead to an increase in genome size⁶. Thus DNA content may evolve or be directly induced under novel environmental conditions.

A rise in the atmospheric concentration of carbon dioxide is an important component of global changes in climate. The frequently reported enhancement of growth of vegetation under higher CO₂ levels⁷ seems to be primarily due to increased availability of CO₂ for photosynthesis or to modulation in the enzymatic activity⁸. We present a survey which suggests that genome size can potentially both influence the responsiveness of a plant species to CO₂ and be affected by elevated CO₂.

Among grasses, the potential advantage of a large genome exists only in the annual species ($n = 7$, $r^2 = 0.876$, $P = 0.002$) and not in the perennial species ($n = 14$, $r^2 = 0.049$, $P = 0.45$) (see figure). Annual grasses not only had greater aver-

age enhancements of growth (ratios of 2.01 compared with 1.27; $P = 0.01$), but also the enhancements were positively correlated with the amount of nuclear DNA. It is not clear why perennial species with large genomes do not show pronounced growth enhancements. Physio-



Relationship between responsiveness to elevated CO₂ and haploid nuclear genome size among annual (black circles) and perennial (open circles) species of grasses. CO₂ responsiveness is calculated as the ratio of average final biomass attained at elevated and ambient CO₂. Linear regression slopes differ significantly (analysis of covariance; $P < 0.0001$); average genome size was similar in both groups. The analysis is limited to grasses due to paucity of the published estimates of both CO₂ effects^{7,10} and measurements of DNA amounts¹ in plants.

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logical adaptations to overwintering or lack of efficient sinks for carbon (in non-reproducing plants) may set constraints on the potential response to an increase in carbon dioxide. There is insufficient evidence to evaluate the potential combined role of genome size, ploidy level, life cycle and phylogeny in other herbaceous species of plants and in trees.

Although multiplication of noncoding DNA comprises most of the increase in genome size, coding regions also become more numerous⁹. The amount of Rubisco or the amount of enzyme responsible for regenerating the supply of ribulose biphosphate, the substrate for Rubisco, may be directly correlated with the number of copies of relevant genes in the nuclear genome. Consequently, a larger genome may be able to support production of larger amounts of Rubisco and counterbalance the CO₂-related reduction in Rubisco activity, thus removing one possible limitation to photosynthesis⁸.

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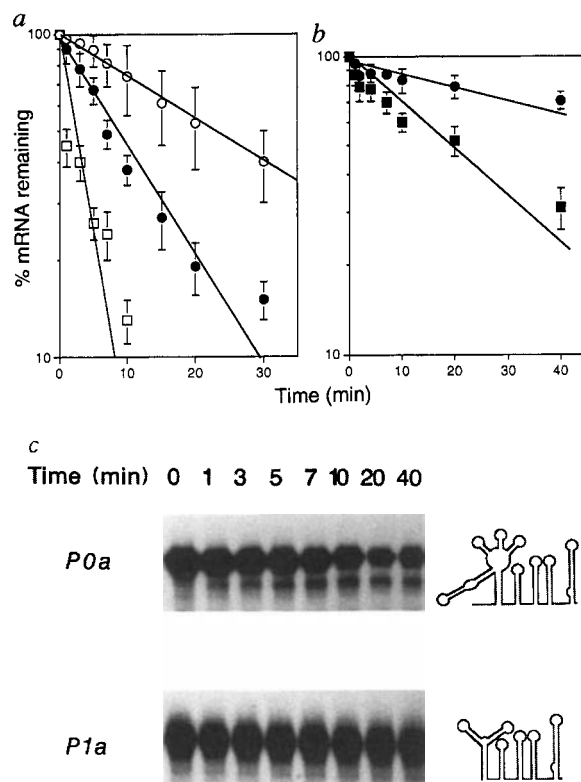
Gene expression and mRNA

SIR—The balance between messenger RNA synthesis and decay has a significant effect on the level of gene expression and the fast turnover of mRNA is crucial for rapid changes in the pattern of gene expression¹. Many bacterial genes have multiple promoters which respond to different signals to facilitate quick adaptation to changes in growth conditions. The selective degradation of mRNAs originating from different promoters in a multiple-promoter gene would enable cells to change the level of gene expression very efficiently and precisely while maintaining a basal level of expression.

The *pts* operon, which encodes several factors in the phosphoenolpyruvate : carboxylate phosphotransferase system (PTS)², is regulated in a complex fashion by at least five promoters, *Pl_a*, *Pl_b*, *P0_a*, *P0_b* and *Px* (ref. 3). The overall expression of PTS is modulated only two- to

threefold, depending on changes in the environment^{3,4}. The mRNAs from *Pl_a* and *P0_a* are two of the major transcripts expressed *in vivo*. The mRNA from *Pl_a* is expressed almost constitutively regardless of the growth conditions whereas *P0_a* expression is increased when cells grow on glucose^{3,4}. The mRNAs from these *pts* promoters have long 5'-untranslated regions (translation starts at +164 from the *Pl_a* start site) with potential secondary structures. The predicted secondary structure of each of the mRNAs has a key difference in the 5' end³. Only the mRNA from *Pl_a* has a stem-loop structure at its 5' end which has been known to protect the mRNA from RNase E, an endonuclease which plays an important role in mRNA degradation in *Escherichia coli*^{1,5,6}.

The degradation of mRNAs from *P0_a* and *Pl_a* was monitored by a primer extension assay after treating the cells with rifampicin to prevent new RNA synthesis. There were large differences in the stability of mRNAs originated from each promoter: the *Pl_a* message was much more stable than the *P0_a* message (*a* in the figure). The half-life of mRNA from *P0_a* and *Pl_a* was below 30 s and 10 min, respectively, in wild-type cells. The stability of mRNA was increased significantly overall in the *rne^{ts}* strain (the mutant strain with heat-sensitive RNase E)⁷ after the heat-sensitive RNase E had been inactivated: the half-life of *P0_a* was increased to 3 min and that of *Pl_a* to 23 min. The *Px* synthesized by σ^{32} RNA polymerase was also stabilized. The effect of RNase E was shown more clearly by an *in vitro* experiment using partially purified RNase E (ref. 8). mRNA from *Pl_a* was more sta-



The differential degradation of mRNAs from *pts* promoters. *a*, Semi-logarithmic graph of *in vivo* mRNA degradation as a function of time. Each point represents the mean of five independent experiments. ○, *Pl_a* mRNA in *rne^{ts}* condition, ●, *Pl_a* mRNA in *rne^{ts}* condition, □, *P0_a* mRNA in *rne^{ts}* condition. *b*, Semi-logarithmic graph of *in vitro* mRNA degradation as a function of time. Each point represents the mean of three independent experiments. ■, *P0_a* mRNA, ●, *Pl_a* mRNA. *c*, The *in vitro* degradation of mRNAs which have the 5'-end regions originating from *P0_a* and *Pl_a* promoters. The predicted secondary structure of each mRNA is shown on the right. The mRNA with the 5'-end region of *Pl_a*, which has a paired nucleotide at the 5' end, is more stable than that from *P0_a*.

ble than *P0_a* (*b*, *c* in the figure), in agreement with the *in vivo* results. A new band appearing above the *Pl_a* band after 7 min (data not shown) and the band below the *P0_a* mRNA (*c* in the figure, top panel) have the same 5' end, indicating that it is a processing product of the *P0_a* transcript.

These results demonstrate the differences in the stability of *P0_a* and *Pl_a* mRNA. The differential degradation of mRNAs produced from multiple *pts* promoters should have a major role in the fine control of *pts* expression. There are clear advantages for cells if they maintain the basal level of *pts* expression through the expression of a stable mRNA from *Pl_a*, while fine modulation of *pts* expression is achieved through the expression of the highly unstable mRNAs from other promoters regulated by various external signals.

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The body in question

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The Body Emblazoned: Dissection and the Human Body in Renaissance Culture. By Jonathan Sawday. Routledge: 1995. Pp. 327. £35, \$45.

Medicine and Society in Later Medieval England. By Carole Rawcliffe. Alan Sutton: 1995. Pp. 241. £25, \$45.

ANY medical student can vouch for the way in which the first few lessons in dissection become etched on the memory. Even with gross anatomy being whittled down in curriculum time, and the advent of newer teaching aids that do not rely on touching a corpse, dissection is still part of the acculturation of the would-be doctor. For centuries, anatomy was considered to be the queen of the medical sciences, but dissection more than any other activity was intimately connected in the public mind with all that was disreputable about medical students. Ruth Richardson's *Death, Dissection and the Destitute* (1988) examined the decades surrounding the passage in Britain of the 1832 Anatomy Act; now, Jonathan Sawday's *The Body Emblazoned* analyses the era of Andreas Vesalius and after. In the years between these two fine monographs, 'the body' has become a fashionable object of historical enquiry.

Sawday approaches his topic as a cultural historian, and although most of the 32 striking plates are taken from anatomical works of his period, he is as much concerned with the language of the poetry of John Donne, Edmund Spenser and Phineas Fletcher as with the anatomical works of Vesalius and Charles Estienne, and with humanistic conceptions of the microcosm as with the functional designs of the anatomy theatres in Padua and Leiden. It is one of the strengths of his study that the obverse is equally true: in this dissection of the culture of science, neither the science nor the culture gets short-changed.

In Greek mythology, Medusa, the only mortal Gorgon, was beheaded by Perseus. Her severed head could still turn to stone anyone who looked at it. The Medusan myth pops up repeatedly in Sawday's volume, as a reminder of the power of dismembered parts and of the latent (or explicit) eroticism in much Renaissance anatomical illustration. Because executed criminals were a main source of supply, women's bodies were especially prized. Female felons could plead the belly, which made pregnant corpses even more valuable. Vesalius is dissecting a pregnant woman in the famous frontispiece of *De Humani Corporis fabrica*, and a number of

Sawday's other illustrations depict mother and fetus.

If Greek mythology provided evidence of the residual power of the dismembered part, Renaissance anatomists first systematically confronted the contrast between the exterior and interior of the human body, and the fact that internal organs are just as individualized as faces and external

emblazon is to celebrate, glorify or praise, as well as to depict heraldically. Several of his anatomical illustrations incorporate heraldic motifs, and both French and English poetical traditions of the period emblazoned female beauty. Nor was it happenstance that literature and anatomy shared a common concern with theatres, spaces where drama was enacted. Renaissance drama, poetry, philosophy and anatomy sought an identical end: *nosce teipsum* — know thyself. To which anatomy added: *nascentes morimur* — we are born to die.

The dustjacket of Sawday's book assures us that his writing is free of jargon. One person's jargon is another person's professional language. Those coming to his book from anatomy may not find his prose as limpid as that which the founders of the Royal Society advocated, but his pages are so full of insight that the effort is rewarded.

By contrast, the hardest bits of Carole Rawcliffe's erudite synthesis in *Medicine and Society in Later Medieval England* are the long quotations in old English. They make scanning difficult, even while serving to reinforce the fact that she is surveying a lost world. Anatomy barely gets a mention, as Rawcliffe examines the ways in which individuals in the fifteenth and sixteenth centuries coped with the uncertainties of existence in this vale of tears. A fine series of illustrations, including 20 in colour, provide more than just decorative attraction to her text, which makes wonderful use of poignant anecdote. Individual chapters on the principal medical orders (physicians, surgeons and apothecaries) are complemented by discussions of women, midwives, the occult, religion and therapy. She enters fully into the values of the society she dissects, explaining why, for instance, prophylactic bloodletting in the spring and autumn could be valued. Monks would have queued up for it, because the ritual surrounding it meant that

they got a few days resting in the infirmary, with a full diet. Sweatings, clysters and purges also had their rationales; so did swaddling newborns.

The nature of sources for understanding health and disease five centuries ago means that Rawcliffe must cast a wide net. Like Sawday, she is not afraid to use literary or theological writings; like him, she demonstrates how impoverished will be the history of medicine if it relies solely on the testimony of doctors. Both volumes eloquently demonstrate the buoyancy of the discipline. □

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Flayed figure from Valverde's Spanish anatomy textbook of 1556, depicting the Marsyas and Apollo myth.

shapes. Sawday minutely examines the Renaissance tradition of illustrating dissected bodies as if they were still alive, relating this to beliefs about the intimacy of mind and body in the pre-Cartesian world. The triumph of the mechanical worldview in the seventeenth century punctured the convention; he argues that only with Sigmund Freud and James Joyce was the older monist position cogently articulated for the modern world. Significantly, Joyce's *Ulysses* — a 'somatic epic' — took some of its inspiration from Fletcher's *The Purple Island* (1633), a once-famous anatomical poem suffused with the mind-body monism of Hippocratic humoralism.

Even the title of Sawday's monograph links the anatomical with the literary. To

Ecological vicars and curate's eggs

John H. Lawton

Species Diversity in Space and Time.

By Michael L. Rosenzweig. Cambridge University Press: 1995. Pp. 436. £50, \$74.95 (hbk); £17.95, \$27.95 (pbk).

A CLASSIC 1895 cartoon in the satirical magazine *Punch* shows a curate taking breakfast with his bishop. "I'm afraid you've got a bad egg, Mr. Jones", the caption reads. "Oh no, my Lord," replies the curate, "I assure you! Parts of it are excellent!"

Species Diversity in Space and Time is a curate's egg. In it, Michael Rosenzweig reviews the literature on patterns in species diversity from local to global scales, and attempts to provide theoretical explanations for the patterns. Some of the theory is familiar, some of it new. The patterns include species-area curves from local to provincial (mainly continental) scales, latitudinal gradients in diversity, species-abundance distributions, changes in species richness through evolutionary time, the relative diversity of polyploid

taxa, body sizes and food webs. Theory, all of it fairly simple and clearly explained, embraces such varied topics as speciation and extinction rates, habitat selection, population dynamics and coevolution. The book is directed at graduate students, and so defines and explains things that established ecologists take for granted. But it is not simply a textbook; it is also a monograph, a personal view of how Rosenzweig sees the world.

I recommend the book for its good parts. They include a clear commitment to explaining to students that the world of ecology is not infinitely wonderful and complex, but can frequently be understood, and its behaviour predicted by relatively simple models. It provides one of the clearest and best accounts of species-area relationships I have read, and it shows how scale matters if we are to understand these relationships. George Gaylord Simpson, for instance, coined the term 'ecological vicars' for pairs of taxa filling similar roles in different biogeographical provinces; their existence is one reason why species-area graphs at continental scales have steeper slopes than within-continental relationships. Between-island slopes lie between these two extremes, and Rosenzweig explains why. He also weaves data from the fossil

record with information from the present in a way that few other ecology texts can match. And he signposts the unknown — both poorly documented patterns, and bold and obvious patterns in nature that we cannot yet explain. To take just one of his top ten problems, why does diversity at regional scales first rise and then fall as productivity increases?

Whether the bad parts spoil the egg is a matter of personal opinion. In the preface, Rosenzweig explains that he has deliberately changed his writing style, and freely admits simply to "dictating a first draft and then abandoning it to the printer". Few are gifted enough to get away with such an approach, and Rosenzweig is not among them. I found the style irritating in too many places, and some of the text and figure legends incomprehensible. If you think hard about it, then the phrase "sink species [that] constantly but unsuccessfully go locally extinct" makes perverse sense, but it is not easy reading.

Before students are let loose on the book they need to be provided with two health warnings. First, not all the data are what they seem. Artefacts and problems of interpretation lurk in many dark corners but are rarely, if ever, discussed. More caution would be wise. Second, coverage of the literature is spotty; brilliant in places, feeble in others. The feeble parts matter, particularly when they provide a distorted view of current understanding. I am flattered, for example, to have more or less a whole chapter devoted to early theoretical work by Stuart Pimm and myself on food webs. But the world has moved on since the late 1970s, and population dynamic models, at best, are now known to provide only partial explanations for patterns in food webs. Similar remarks apply to the ratio of predator species to prey species, and to several other parts of the text.

The curate ate his egg under sufferance, too frightened to complain. I am not so constrained, and have probably complained too much, because despite its problems I nevertheless enjoyed the book. I read it, incidentally, sitting in a tropical forest in Cameroon, surrounded by an almost bewildering diversity of plants, insects and birds. Rosenzweig's fundamental explanation for that diversity is habitat area; latitudinal gradients must arise because the tropics cover more area than any other zone. The hypothesis is bold, simple and convincing, and typifies the book's good parts. I will recommend *Species Diversity in Space and Time* to my graduate students to read because the good bits outweigh the bad. It will give us much to think about, and should generate some lively debates. □

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THE marsh and estuary of the Great Bay/Piscataque River are some of the most ecologically important and sensitive regions in the Gulf of Maine, New England. The picture is one of many stunning satellite images and aerial photographs reproduced in *From Cape Cod to the Bay of Fundy: An Environmental Atlas of the Gulf of Maine* edited by P. W. Conkling. MIT Press, \$50, £42.50 (hbk), \$29.95, £25.50 (pbk).

European science and public policy

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Uno Spazio Europeo della Scienza: Riflessioni sulla Politica Europea della Ricerca. By Antonio Ruberti and Michel André. Giunti Editore: 1995. Pp. 162. L24,000 (pbk).

MODERN science is marked by extraordinary growth: 90 per cent of all the scientists and technologists who have populated human history are alive and working today, and three million articles appear in scientific publications each year. Our 'scientific system' is characterized by many different objectives and interactions that render it extremely complex. Despite the fact that complexity theorists are now dealing with at least 31 different definitions of complexity, over recent decades the characteristics of the scientific system have been subjected to ever-more detailed studies.

This book appears at a crucial moment in the development of the research world: by establishing the boundaries of its various areas, identifying possible overlaps and emphasizing the principal dynamic traits, the authors dispel much of the confusion that, until now, has prevented parameters and concepts from being clearly understood. The book focuses on Europe and outlines what would constitute an authentic European public research and development policy. Needless to say, frequent comparisons with the United States and Japan and references to action in favour of developing countries are also included. It should therefore also enjoy a wide readership outside Europe.

Unified policy

The authors begin with an analysis of the past and present, providing an overview of the mechanisms that, since the end of the Second World War, have gradually been created to promote scientific cooperation among European countries. They view the diversity of traditions and the broader social and cultural aspects of scientific activity as the starting points for a unified European research policy.

They point out that alongside the economic and political space that dominated the initial phases of European unification, a new intellectual scientific and cultural space has now developed on a continental scale. This new space not only lends support to the unification process but also lays the groundwork for

its future development. The authors emphasize that during world history in general and European history in particular, the circulation of ideas and the circulation of goods have always gone hand in hand, and that the two processes have been self-catalysing. The space of European science is therefore a truly 'open' one that cannot but take into account changes in Eastern Europe, especially in the light of the considerable scientific and technological patrimony of that region.

The authors' proposals for the future take their cue from the 'white book' (an analogy with a white paper or policy document) entitled *Growth, Competitiveness and Employment* that the European heads of state and of government officially approved in December 1993. The book identifies three faults in the European research system. The first is inadequate resources. Europe invests proportionally less than its competitors in research and development. This is especially true for companies, which are usually impatient and reluctant to get involved in fundamental research that, by its very nature, is many years removed from potentially marketable products.

The second fault is inefficiency in the transformation of scientific progress and technological innovation into industrial and commercial successes. This is primarily the result of organizational, cultural and social factors. It is enough to imagine the difficulties — the lack of incentives and the presence of regulatory obstacles — encountered by a European researcher who sets about creating a company.

Finally, the third fault is fragmentation, dispersion and insufficient coordination. Unlike the United States or Japan, Europe has 15 or 18 or 20 different national research policies, depending on how one looks at them: the policies of the European Union, those of the large organizations of scientific cooperation such as CERN (the European Laboratory for Particle Physics) and those of the different countries in the European Union.

These weak points present a tremendous challenge for the European system. But what resources can it rely on? What can be done to increase them? How can existing resources be put to best use?

The European Union's annual research budget is ECU2.8 billion. This is less than 4 per cent of the public expenditure on research of the countries concerned. If one includes the funding that national research organizations set aside to support the participation of their research teams in European Union programmes, the national funding of the Eureka project and the endowments of the large cooperative organizations such as CERN, the European Space Agency

(ESA), the European Space Organization (ESO) and the European Molecular Biology Laboratory (EMBL), one reaches a figure that is about 13 per cent of Europe's total expenditure for research. (Initiated by President François Mitterrand of France in response to the US Strategic Defense Initiative, the Eureka project is based on the 'à la carte' shares principle, lacks independent resources and finances its activities with funds from the various national governments.)

Investment returns

Despite the fact that economic parameters in general indicate that a period of recovery and expansion has already begun, it seems unlikely that there will be any increase in investment in research in the short term. Governments are debating how much money to allot to research and the rationale behind their allocation of resources.

There is no doubt that science brings great benefits to society. According to Laura Tyson, chairperson of the President's Council of Economic Advisors, basic research in the United States yields an annual rate of return on investment ranging from 20 to 50 per cent. This is enormous compared with the rates of return of other organized endeavours. These returns on investment apply to both academic and industrial research; but one should not forget that with academic research, new human capital is also generated.

If investment in research is so productive, why are governments and industries reluctant to increase it? In today's climate of budgetary restrictions, there is a tendency to believe that balancing budgets will guarantee a safe future for the next generation. It would be more appropriate to re-examine investment in research; the public and politicians should be aware that increasing resources for research, especially for fundamental research, means making a wise, long-term investment in their countries' future. Given the present situation, what is crucial is that the resources available are well spent.

The goal of a unified research policy obviously does not, and indeed should not, imply complete centralization. Rather, it should enhance cooperation and complementarity. A simple rule would be to consider a European Union institution or initiative to be worthwhile only if the advantages it brings to the national communities are vastly greater than those attained by the different countries acting independently. For example, with the introduction of gene knock-out technology in Europe, at least a thousand mouse mutants are produced each year, a figure that is bound to increase rapidly. There is no laboratory with the means to maintain

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and distribute many mutant mice; but without such a facility, the mutants run the risk of being irretrievably lost. The recent decision to create the European Mutant Mouse Archive is an example of the application of the rule proposed above.

Existing cooperative programmes could be improved by keeping past experience in mind. The administration in Brussels must not be left alone with such a task, not least because the language used in its documents is vastly different from that of contemporary science. (I am of the opinion that the responsibility for the administration's isolation lies mostly on the shoulders of European scientists.)

The recently instituted European Sciences and Technologies Assembly (ESTA) could become the tool for improving the European Union's research and technological development policies while strengthening coordination of the research policies and activities of

European countries. The role played by ESTA, *mutatis mutandis*, should be analogous to that of the US National Research Council. ESTA brings together scientists who are supposed to guarantee contacts with the frontiers of research and development, the directors of European research organizations such as EMBL, ESO and so on, the presidents of national research organizations and the heads of the research departments of large industrial concerns. It is not at all rash to conclude that the possibility of realizing a Unitary European Research and Technology Policy depends entirely on how ESTA handles the tasks assigned to it. As ESTA was created during Ruberti's term as commissioner, how it actually fares in practice will determine the outcome of Ruberti's and André's reflections. □

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Fleshing out intelligent machines

Igor Aleksander

The Handbook of Brain Theory and Neural Networks. Edited by Michael A. Arbib. MIT Press: 1995. Pp. 1,118. \$150, £95.

It must say something about the science of neural networks that it has gone from being a fringe activity for the misguided few to meriting Michael Arbib's encyclopaedic treatment in the space of 12 or so years. At more than three kilograms, this handbook is twice the weight of an adult brain and far beyond the size of the average book one might read on the train. It claims to tackle the questions "How does the brain work?" and "How can we build intelligent machines?" through its 266 articles written by individuals and a few guiding introductions written by the editor and his advisers. It should immediately be said that the articles on brain modelling are of a 'computational' kind: that is, they concentrate on theoretical models rather than reporting on neurobiological findings.

Can one really learn about brains by doing mathematics and simulating artificial neural networks? In a very broad sense the answer must be yes. A neural network is a system containing elements that adjust their function according to some external requirement. The brain too is a network of variable-function cells and in this sense has the artificial neural network as its distant cousin. Studying the artificial network is more tractable than studying the brain, and the former leads to articles in the handbook on epileptic nets, nets that sort visual back-

ground from foreground and nets that act like the hippocampus. The credibility gap comes from the fact that many artefacts such as error back-propagation are unlikely to exist in the brain. Also, many theoretical models are used because of their mathematical elegance rather than their biological relevance. Personally, I would be happier if the neural network community were more realistic about the true biological relevance of their work and stopped believing that if an artificial network behaves in a brain-like way, the exact underlying mechanism must somewhere to be found in the flesh. The true biological significance of neural modelling lies in the discovery of broad principles and not detailed function. My main criticism of the handbook is that it makes little mention of what is known of such principles — the role of network topology, the structure-function problem, the richness and plasticity of mental states, the constraints of evolutionary development, the difference between learning, adaptation and instruction, the role of modularization, and so on.

So what of intelligent machinery? I happen to believe that this is really where neural networks are now making an impact. Sadly, it too is not a strong point of this handbook. Although applications are mentioned in a sensible article by Françoise Fogelman-Soulie, the handbook gives the impression that the biological effort is paramount and that the engineering of neural systems is a lesser pursuit. Many important engineering contributions such as the work on sparse

memory by the Finnish engineer Pennti Kanerva and the UK-based work on logical nets receive no mention at all.

The overall coverage can be best understood by looking at the main sub-headings: connectionism and artificial intelligence, dynamics, the mathematics of learning, applications, models of biological neurons, sensory systems, biological learning and motor control. As with many handbooks of this kind, the contributions are useful but should be treated with care. The detailed articles, while providing a reasonably extensive coverage, are written by experts describing their own research. In many, an attempt has been made to provide a good perspective and there are some good, informative reviews such as Paul Werbos's treatment of back-propagation (training of multi-layer networks) and Jeffrey Elman's article on language processing. On the other hand, Eduardo Sontag's paragraphs on automata and neural networks hardly touch on the considerable literature now available on the topic. There are sins on the neurobiological side too. Material on models of the hippocampus makes no reference to important work at the University of Oxford in the United Kingdom and at Rutgers University in the United States. Driven by my own interest I looked up the article on "Theories of Consciousness" by Max Velmans — interesting but hardly comprehensive. There is virtually no reference to the intricacies of the current debate on this topic or to the vast philosophical history of the subject. This is merely an example of the real dangers of trying to gain entry into a complex element of the widespread field of neural networks by reading an article of three or so pages.

How permanent is the content of the handbook? Regrettably, the field is not yet sufficiently mature to provide stability, for reasons already mentioned. But, despite this negative impression, for a year or two this book will be a handy addition to the library shelves of any institution in which research is being done on neural networks. The number of contributions and the expertise of the contributors are reasonable. It is the patchiness, the lack of treatment of general principles and the lack of reference to many applied machine-building techniques that make the project less effective than it might have been. Many publishers have embarked on similar projects and other tomes in the pipeline may plug some of the holes. So, librarians, strengthen your shelves in the vicinity of the "neu-" areas. □

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New four-million-year-old hominid species from Kanapoi and Allia Bay, Kenya

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Nine hominid dental, cranial and postcranial specimens from Kanapoi, Kenya, and 12 specimens from Allia Bay, Kenya, are described here as a new species of *Australopithecus* dating from between about 3.9 million and 4.2 million years ago. The mosaic of primitive and derived features shows this species to be a possible ancestor to *Australopithecus afarensis* and suggests that *Ardipithecus ramidus* is a sister species to this and all later hominids. A tibia establishes that hominids were bipedal at least half a million years before the previous earliest evidence showed.

THE recently described early hominid *Ardipithecus ramidus*, from Aramis, Ethiopia, extends the temporal distribution of known fossil hominids to 4.4 Myr^{1, 3}. The relationship between *A. ramidus* and *Australopithecus afarensis* from Hadar, Ethiopia, and Laetoli, Tanzania, (3.0–3.6 Myr) remains speculative, and so the recent recovery of hominid specimens from Kanapoi, Kenya, more than 4 Myr old, helps to test the hypotheses that *A. ramidus* represents a potential root species for the Hominidae¹ and that there was prolonged stasis within *A. afarensis* between 3.9 and 3.0 Myr⁴.

Description of *Australopithecus anamensis*

Order Primates Linnaeus 1758
Suborder Anthroidea Mivart 1864
Superfamily Hominoidea Gray 1825

Australopithecus DART 1925

Australopithecus anamensis sp. nov.

Etymology. The name 'anam' means 'lake' in the Turkana language. All specimens found so far are from near present Lake Turkana and were found in sediments associated with the ancient precursor lake, Lake Lonyumun.

Types. The holotype is KNM-KP 29281 (Fig. 1), a mandible with all teeth, but lacking rami, found by Peter Nzube in 1994. A partial left temporal is probably from the same individual. The paratypes are listed in Table 1. The repository is the National Museums of Kenya, Nairobi.

Localities. Kanapoi localities are situated about 36°04' E and 2°19' N between the Kalabata and Kakurio rivers in Turkana District, northern Kenya (Fig. 2). Specimens from East Lake Turkana come from Site 261-1 and a nearby locality 1 km to the northeast in Area 261. Locations of individual fossils are marked on aerial photographs housed in the National Museums of Kenya.

Horizon. The type is from Pliocene strata at Kanapoi, between the lower and upper pumiceous tephra (about 4.1 Myr). Paratypes from Kanapoi come from this level or slightly higher in the section while those from Allia Bay lie below or within the Moiti Tuff (about 3.9 Myr) in the Koobi Fora Formation.

Diagnosis. A species of *Australopithecus* distinguished from all others by the following features: a small external acoustic meatus presenting a narrow ellipse in outline; long axes of mandibular bodies and tooth rows nearly parallel and close together; mental region of mandible not strongly convex; long axis of symphysis slopes markedly posteroinferiorly; canines with very long robust

roots; trigons of upper molars much wider than talons; distal humerus with thick cortex enclosing small medullary cavity. It can be distinguished from *A. afarensis* by the following: upper canine root and associated facial skeleton less posteriorly inclined; lower molars tend to have more sloping buccal sides and upper molars more sloping lingual sides; tympanic plate very horizontal without defining grooves. It can be distinguished from *Ardipithecus* by the following features: absolutely and relatively thicker tooth enamel; upper canine buccal enamel thickened apically; molars more buccolingually expanded; first and second lower molars not markedly different in size; tympanic tube extends only to the medial edge of the postglenoid process, rather than to the lateral edge or beyond it; lateral trochlear ridge of humerus weak.

Dental and cranial descriptions

The sample shows some size variation in teeth and mandible that can probably be attributed to sexual dimorphism (Table 1). The type mandible, which lacks the rami, has a relatively small body, but the fragment, KNM-ER 20432, and some pieces associated with the lower teeth, KNM-KP 29286, are both larger, particularly in depth, in the anterior part of the body. The canine in the type mandible is smaller than those in the other two. The type mandible is remarkable in that the long axis of the symphysis is very posteriorly inclined, so that the mental surface extends as far posteriorly as the first molar and curves only slightly between the incisor alveolae and the inferior torus. There is a very long postincisive planum together with a moderately developed superior torus and a well-developed inferior torus. The body becomes everted posteriorly and X-rays reveal a serrate root-pattern⁵, as seen in other early hominids. This specimen is slightly distorted by the forward displacement of the left second incisor and canine roots from their alveoli. *A. anamensis* teeth are similar to those of *A. afarensis*, especially those from Laetoli, with the following major differences. Among the lower teeth: the *A. anamensis* canine is larger in size and usually more asymmetrical with a long robust root; the third premolar is very asymmetrical with a blunt centrally positioned main cusp and with clearly separated mesial and distal roots; the third and fourth premolars have extensive sloping buccal surfaces, and the molars tend to have more sloping buccal sides. Among the upper teeth: the molars of *A. anamensis* have trigons much wider than talons; the canines have long robust roots and crowns with apically thickening enamel which is ~1.3 mm closer to the apex, and similar to *A. afarensis*, but greater than that of *A. ramidus* (>0.9 mm)¹. Naturally cracked upper and lower molars have

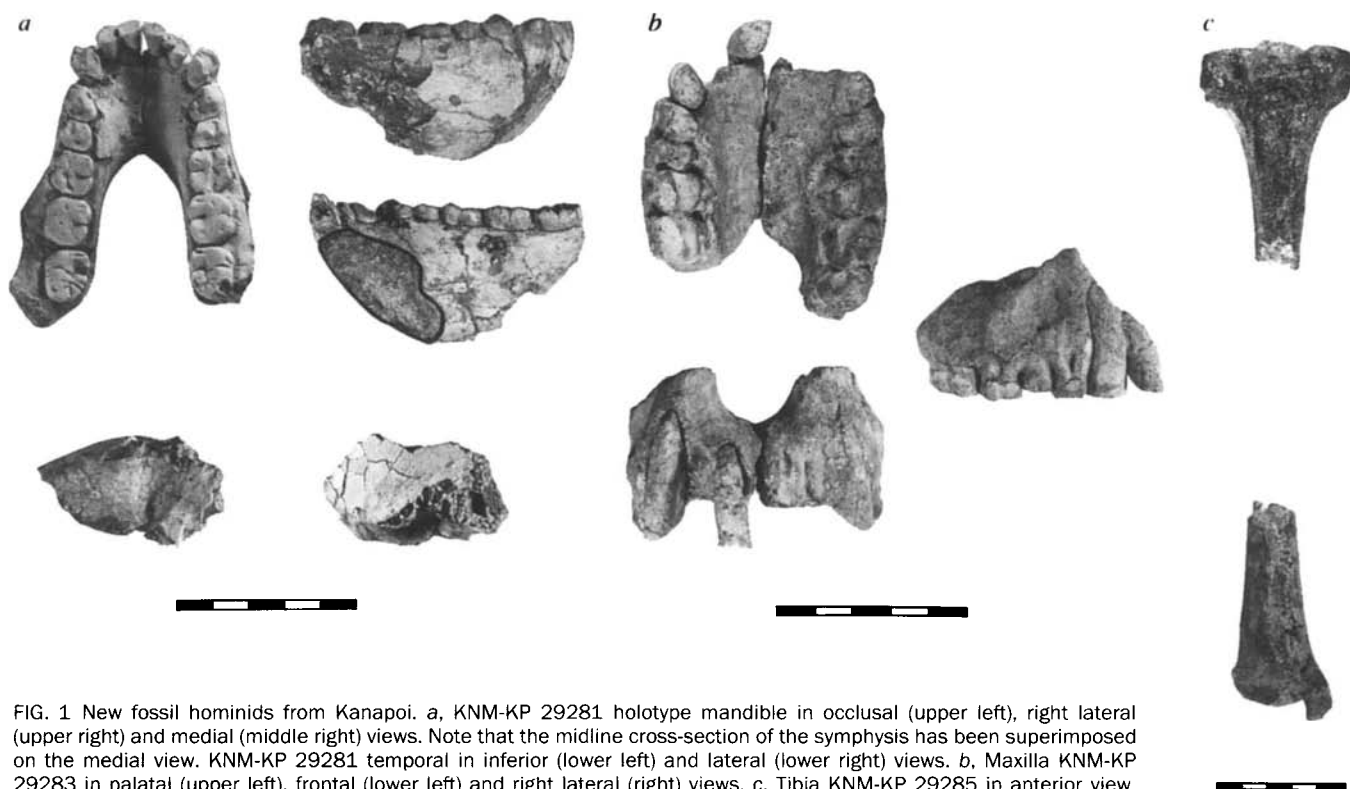


FIG. 1 New fossil hominids from Kanapoi. a, KNM-KP 29281 holotype mandible in occlusal (upper left), right lateral (upper right) and medial (middle right) views. Note that the midline cross-section of the symphysis has been superimposed on the medial view. KNM-KP 29281 temporal in inferior (lower left) and lateral (lower right) views. b, Maxilla KNM-KP 29283 in palatal (upper left), frontal (lower left) and right lateral (right) views. c, Tibia KNM-KP 29285 in anterior view. Scale bars are in cm.

enamel as thick as that reported for *A. afarensis*, and thicker than that of *A. ramidus*; enamel thickness near cusp apices can be measured between 1.5 and 2.0 mm ($n=9$), almost exactly matching the range reported for similar measurements at uncontrolled locations in *A. afarensis*, and about twice that reported for *A. ramidus*.

Part of a left temporal bone is probably associated with the type mandible. It consists of the lower part of the squamous portion, the tympanic and most of the mandibular fossa. The tympanic is flat inferiorly and set at a very high angle to the roof of the mandibular fossa. The entoglenoid process is incomplete, but clearly small, and the articular eminence very slight. The squama and the root of the zygomatic process are pneumatized. The external acoustic meatus is small (8.9 mm vertically by 5.7 mm anteroposteriorly with an area of 50.7 mm²) and its shape in outline, a vertically oriented ellipse, is most often found in chimpanzees. African apes have small meatuses (areas for *Pan troglodytes*, $\bar{x}=43.7$ mm², s.d. 10.5, $n=21$; for *Gorilla gorilla*, $\bar{x}=53.9$ mm², s.d. 18.5, $n=21$) whereas humans and *A. afarensis* have large meatuses (areas for *Homo sapiens*, $\bar{x}=115.1$ mm², s.d. 17.3, $n=22$; for *A. afarensis*, $\bar{x}=97.9$, $n=5$ (W. H. Kimbel, personal communication)). It must be noted, however, that the sample from Hadar is extremely variable, ranging from 44 to 139.2 mm². Two *A. ramidus* specimens have small meatuses (T. D. White, personal communication). The tympanic of the Kanapoi specimen also lacks the deep furrows bounding it anteriorly and posteriorly that are described for *A. ramidus*¹. The maxilla, KNM-KP 29283, together with KNM-ER 30200, which has relatively unworn molars, shows several differences from later *A. afarensis*. The canine root in *A. anamensis* is placed more vertically, and because of this the lower lateral margins of the piriform aperture appear to be more vertical. Note that the Garusi specimen, a small maxillary fragment from Laetoli⁹, is very similar in this respect to KNM-KP 29283. It also preserves a small part of the midline suture⁷, showing that the palate was very shallow and narrow, as is also the case in KNM-ER 30200. The upper molars in *A. anamensis* have more lingual flare and are

wider mesially than distally, which gives them a posteriorly wedged outline, whereas they are more square in *A. afarensis*.

Postcranial description

The tibia, KNM-KP 29283, is larger than the largest from Hadar (A.L. 333-42). The middle of the shaft was not recovered. We estimate that between a third and a half of the total length of the bone is missing. Using regression equations based on humans⁸, the estimated body weight of this individual is 55 kg (based on the upper epiphysis) and 47 kg (based on the distal epiphysis). This is somewhat higher than the mean body weight recently estimated for the males of *Australopithecus afarensis*, and 1.7 times higher than that computed for presumed females⁸. The most important features of this bone are those that indicate bipedalism^{9,10}. These include: rectangular proximal surface with anterior/posterior lengthening of the articular surfaces, condyles both concave and of roughly equal area, expanded metaphyseal bone, probably small fibular articulation, very straight shaft in those parts preserved, and a distal articular surface that faces directly inferiorly. Although the proximal fibular facet is broken away, the small size of the missing area shows that the articulation must also have been small, as in humans. We conclude that bipedal locomotion had evolved at least half a million years before the previous earliest evidence (the footprints at Laetoli) suggests. There are a few primitive features of this bone. These include a projecting attachment for the fascia lata, a less swollen superior metaphysis, and a strongly marked gracilis insertion next to the anterior border of the shaft. In these respects the specimen resembles African apes and *A. afarensis*. The distal humerus, KNM-KP 271, was originally seen to be human-like^{11,14}, and it does show many derived hominid features, including a marked median anterior capsular ligament tubercle (I. Hershkovitz, personal communication).

Discussion

This new species of *Australopithecus* has a mixture of features that are both primitive and derived for the Hominidae. Of par-

TABLE 1 Fossil hominids from Kanapoi and Allia Bay

(a)				
Specimen number	Date	Site	Body part	Discoverer
KNM-KP 271	1965	Kanapoi	Distal left humerus	B. Patterson
KNM-KP 29281	1994	Kanapoi	Holotype mandible with left and right I ₁ -M ₃ and probably associated partial left temporal bone	P. Nzube
KNM-KP 29282	1994	Kanapoi	M ₂	P. Nzube
KNM-KP 29283	1994	Kanapoi	Maxilla with right I ¹ , C-M ² and left C-M ³ , with associated left I ₂	W. Mangao
KNM-KP 29284	1994	Kanapoi	Associated lower right C and P ₃ germs	Sieving team
KNM-KP 29285	1994	Kanapoi	Right tibia lacking middle half-third of shaft	K. Kimeu
KNM-KP 29286	1994	Kanapoi	Associated lower dentition, lacking right I ₁ with fragments of mandibular body	P. Nzube
KNM-KP 29287	1994	Kanapoi	Right and left mandible fragments with molar roots	S. Ngui
KNM-KP 29288	1994	Kanapoi	Tooth fragments and partial upper C	Sieving team
KNM-ER 7727	1982	Allia Bay 261-1	left M ²	J. Kithumbi
KNM-ER 20419	1988	Allia Bay 251	left radius	M. Kyeve
KNM-ER 20420	1988	Allia Bay 261-1	left M ²	J. Kimengich
KNM-ER 20421	1988	Allia Bay 261-1	right M ³	Sieving team
KNM-ER 20422	1988	Allia Bay 261-1	left M ₁	Sieving team
KNM-ER 20423	1988	Allia Bay 261-1	left M ₂	Sieving team
KNM-ER 20427	1988	Allia Bay 261-1	left M ¹	Sieving team
KNM-ER 20428	1988	Allia Bay 261-1	left M ₃	Sieving team
KNM-ER 20432	1988	Allia Bay 261-1	left mandible fragment with canine root and P ₃₋₄	Sieving team
KNM-ER 22683	1988	Allia Bay 261-1	left P ₄	Sieving team
KNM-ER 24148	1988	Allia Bay 261-1	left dm ²	Sieving team
KNM-ER 30202	1995	Allia Bay 261-1	right I ¹	A. Walker
KNM-ER 30200	1995	Allia Bay 261	left maxilla with M ¹⁻³	K. Kimeu
(b)				
Specimen number	Side	Tooth	Mesiodistal diameter (mm)	Buccolingual diameter (mm)
KNM-KP 29281	L	I ₁	6.0	7.5
	R	I ₁	6.1	7.5
	L	I ₂	6.4	8.7
	R	I ₂	6.7	8.7
	L	C	(9.0)	9.6
	R	C	9.6	10.9
	L	P ₃	10.6	9.6
	R	P ₃	9.8	10.9
	L	P ₄	8.4	10.0
	R	P ₄	8.2	10.6
	L	M ₁	12.0	11.9
	R	M ₁	12.1	12.0
	L	M ₂	13.0	12.7
	R	M ₂	13.2	12.5
	L	M ₃	14.5	12.4
	R	M ₃	14.8	12.3
	R	I ¹	8.4	8.6
KNM-KP 29283	R	C	11.7	9.2
	L	P ³	9.0	12.6
	R	P ³	8.9	11.6
	L	P ⁴	7.7	11.7
	L	M ¹	(11.0)	(13.0)
	L	M ²	13.5	
	R	M ²	13.5	14.8
	L	M ³	12.7	13.8
KNM-KP 29284	R	C	13.0	9.1
	R	P ₃	10.9	9.6
KNM-KP 29286	L	I ₁	6.6	
	L	I ₂		7.8
	R	I ₂		7.7
	R	C	9.2	11.7
	L	P ₃	9.9	12.4
	R	P ₃	9.6	12.0
	L	P ₄	9.7	11.7
	R	P ₄	9.6	11.6
	L	M ₁	12.3	12.0
	R	M ₁	12.3	12.2
	L	M ₂	14.6	13.7
	R	M ₂	14.6	13.6
	L	M ₃	14.4	12.8
	R	M ₃	13.8	12.3
KNM-ER 30200	L	M ¹	10.2	11.9
	L	M ²	11.5	13.2
	L	M ³	(11.0)	(12.0)
KNM-ER 30202	R	I ¹	10.5	9.0

(a) List of fossil hominids from Kanapoi and Allia Bay. The Allia Bay hominids, with the exception of KNM-ER 30200 and ER 30202, have been described by Coffing *et al.*¹⁷. (b) Tooth measurements of new Kanapoi and Allia Bay hominids. Measurements for other Allia Bay teeth are given in Coffing *et al.*¹⁷.

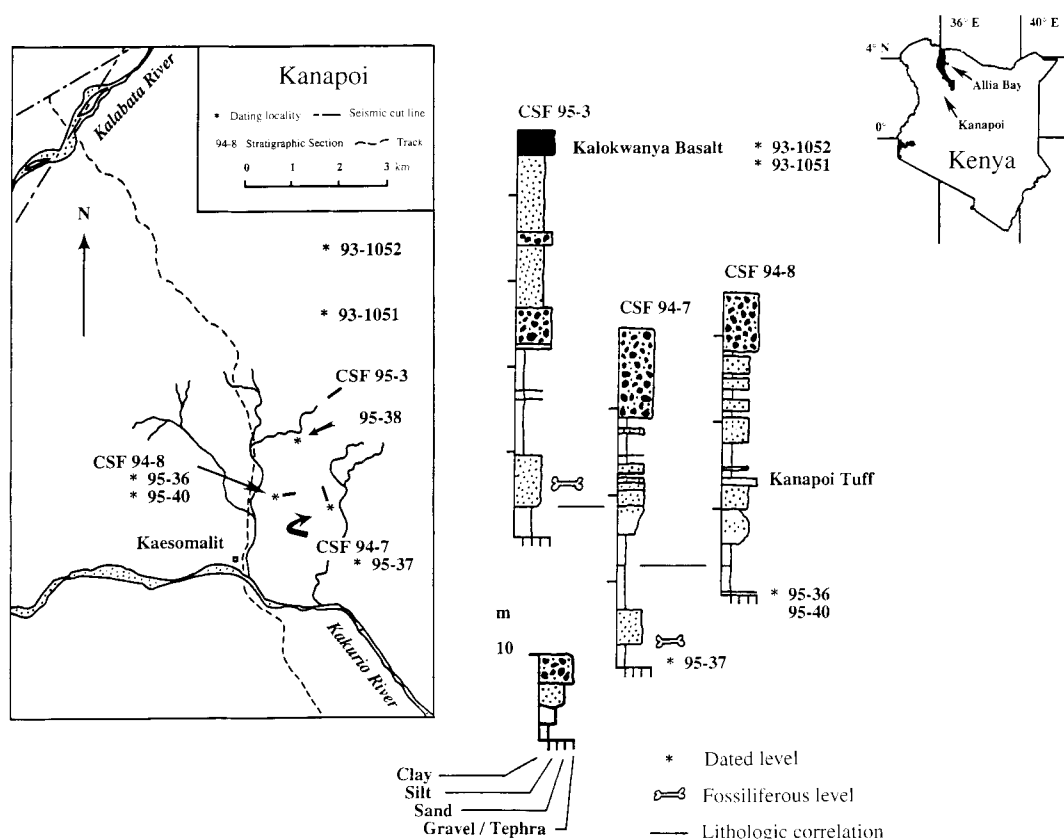


FIG. 2 Location maps and stratigraphic sections of the Kanapoi area. Geographic context of stratigraphic sections and dating samples, and lithostratigraphic sections showing relationship of fossiliferous units and dating samples. In the basal claystone unit of section CSF 94-8, small altered clasts (probably former pumices) were found to contain relatively fresh alkali feldspar crystals up to 1 mm in size. Samples from this level were used to prepare feldspar concentrates 95-36 (=K94-5401),

95-40 (=K95-5501). A third sample from the same horizon at another locality produced concentrate 95-38 (=K94-5490). Results of total fusion ^{40}Ar - ^{39}Ar dating of 8 single crystals from each of these samples are given in Table 2. In section CSF 94-7, a basal claystone unit also was found to contain small altered clasts, probably originally pumices. A bulk separate of alkali feldspar from this horizon, 95-37 (=K94-5385), provides supporting age control (Table 2).

ticular note are the *Homo*-like features of the tibia which complement those already noted for the Kanapoi humerus. This latter is quite large, from an individual judged by regressions based on humans to have weighed about 58 kg⁸. *A. anamensis* is seen as a possible ancestor to *A. afarensis*, although it is recognized that 4 Myr ago there may have been a number of newly emergent hominid species with variations based on the novel bipedal adaptation.

It should be noted that the *A. afarensis* hypodigm was collected from two localities, Laetoli and Hadar, which are ~1,500 km apart. *A. anamensis* can be readily distinguished from the younger Hadar sample, but has closer affinities with the older Laetoli specimens. In particular, similarities are seen in the very large size of the canines, the accessory distal cuspules of the lower canines, the exaggerated flare on the molars, the tapering upper molars and the vertically implanted upper canines. It must be noted, however, that the Laetoli sample is small, consisting almost entirely of parts of mandibles and isolated teeth and with no adult postcranial bones. Based on a fragmentary frontal from Belohdelie and a new skull from Hadar, Kimbel *et al.*⁴ suggested a prolonged stasis in *A. afarensis* between 3.9 and 3.0 Myr ago. These additional specimens from the early time period show both similarities to and considerable differences from the later Hadar sample. Given that mosaic evolution appears common in the Hominidae, and that there is no theoretical reason why there should be only one early hominid species at any one time, we feel that the case for overall stasis in the hominid skeleton for a million years might have been overstated. Detailed compari-

sons with *A. ramidus* cannot yet be made, but several features clearly distinguish it from *A. anamensis*. At present we know of no characters that would preclude *A. ramidus* from a position as the sister taxon to all other hominids.

The differences between *A. anamensis* and *A. ramidus* in tooth proportions and enamel thickness, together with temporomandibular joint differences such as postglenoid process position, probably indicate differences in food processing and/or food material properties. The recent discovery of a mandible of *Ardipithecus*² will allow this idea to be tested.

Geological context and dating

The Kanapoi sedimentary sequence (Fig. 2) includes three stratigraphic intervals. A basal sequence, deposited on a dissected volcanic landscape, includes conglomerates, sandstones and claystones indicative of a fluvial depositional environment. These strata are overlain by a lacustrine interval of claystones, molluscan packstones and an upward-coarsening deltaic cap. Succeeding strata indicate a return to fluvial conditions, with sandstones, claystones and several conglomeratic channels. These are capped by the Kalokwanya basalt.

Two tephra-bearing horizons occur within the lowermost interval of the Kanapoi sequence. Both are brown claystones containing light-coloured, altered clasts, probably former pumices, up to about 10 mm across. Vitric components in these altered clasts have been replaced by clay and zeolite minerals, leaving a residual population of alkali feldspar crystals ranging up to about 1 mm in size, suitable for ^{40}Ar - ^{39}Ar age determina-

TABLE 2 Results of single crystal dating

Sample	Mass (mg)	$^{36}\text{Ar}/^{39}\text{Ar}$ $\times 10^{-4}$	$^{37}\text{Ar}/^{39}\text{Ar}$ $\times 10^{-2}$	$^{40}\text{Ar}/^{39}\text{Ar}$	K/Ca	$^{40}\text{Ar}^*/^{39}\text{Ar}_K$ $\pm \text{c.v.}$	$^{40}\text{Ar}^*$ (%) (10^{-14} mol)	^{39}Ar	Calculated age Myr ± 1 s.d.
93-1081A (K93-6024); Moiti Tuff pumice, Lokochot Cliff site, aerial photograph 1699/135-132, Koobi Fora; $J=0.001182 \pm 0.30\%$; level 10, ANU4/211									
crystal 1	0.8	7.782	0.866	2.091	60.8	$1.837 \pm 0.76\%$	87.9	1.14	3.912 ± 0.032
2	0.8	11.96	0.715	2.187	73.6	$1.810 \pm 0.64\%$	82.7	1.20	3.854 ± 0.027
3	0.6	5.935	0.512	2.048	102.8	$1.848 \pm 0.65\%$	90.3	1.04	3.935 ± 0.028
4	1.0	5.656	0.635	2.040	82.9	$1.849 \pm 0.43\%$	90.6	1.48	3.938 ± 0.021
5	1.1	3.734	0.848	1.974	62.0	$1.840 \pm 0.63\%$	93.2	1.63	3.917 ± 0.027
6	1.5	4.030	0.622	2.005	84.6	$1.862 \pm 0.36\%$	92.9	2.05	3.965 ± 0.018
7	1.7	3.245	0.697	1.982	75.6	$1.862 \pm 0.31\%$	94.0	2.33	3.964 ± 0.017
8	1.2	13.45	0.695	2.269	75.8	$1.848 \pm 0.82\%$	81.4	1.50	3.934 ± 0.034
9	0.8	14.44	0.635	2.272	82.9	$1.822 \pm 0.65\%$	80.2	1.10	3.879 ± 0.027
					$\bar{x}_9$	77.9 ± 12.7			$\bar{x}_9$ 3.922 ± 0.037
95-36 (K94-5401); upper pumiceous tuff, section CSF 94-8/1, 0027/160-102, Kanapoi; $J=0.001176 \pm 0.30\%$; level 3, ANU4/211									
crystal 1	1.1	2.371	2.082	2.038	25.3	$1.940 \pm 0.51\%$	95.2	1.66	4.112 ± 0.024
2	2.0	2.547	2.235	2.046	23.5	$1.943 \pm 0.47\%$	94.9	2.65	4.118 ± 0.023
3	1.0	2.800	2.007	2.052	26.2	$1.941 \pm 0.46\%$	94.6	1.72	4.114 ± 0.022
5	1.2	3.036	0.476	2.067	110.6	$1.947 \pm 0.72\%$	94.2	1.85	4.127 ± 0.032
7	0.8	3.275	2.369	2.070	22.2	$1.945 \pm 0.52\%$	94.0	1.20	4.123 ± 0.025
8	1.0	3.008	1.981	2.061	26.6	$1.944 \pm 0.79\%$	94.3	1.01	4.121 ± 0.035
9	1.1	9.475	10.04	2.155	5.2	$1.944 \pm 0.40\%$	90.2	1.75	4.120 ± 0.021
					$\bar{x}_7$	34.2 ± 34.5			$\bar{x}_7$ 4.119 ± 0.005
4	1.9	1.265	2.019	2.042	26.1	$1.976 \pm 0.27\%$	96.8	3.62	4.189 ± 0.017
95-40 (K95-5501); upper pumiceous tuff, section CSF 94-8/1, 0027/160-102, Kanapoi; $J=0.001180 \pm 0.30\%$; level 9, ANU4/211									
crystal 2	0.7	3.131	1.857	2.056	28.3	$1.940 \pm 0.65\%$	94.4	1.01	4.124 ± 0.030
3	0.7	2.497	1.098	2.028	47.9	$1.930 \pm 0.47\%$	95.2	1.28	4.103 ± 0.023
4	0.6	4.004	1.759	2.065	29.9	$1.923 \pm 0.64\%$	93.1	1.17	4.088 ± 0.029
5	1.2	6.152	2.106	2.150	25.0	$1.945 \pm 0.57\%$	90.5	1.75	4.135 ± 0.027
6	1.4	1.278	2.061	2.018	25.5	$1.957 \pm 0.40\%$	97.0	1.98	4.161 ± 0.021
7	1.5	1.078	2.355	2.002	22.3	$1.947 \pm 0.39\%$	97.3	2.49	4.139 ± 0.020
8	1.5	11.83	2.165	2.305	24.3	$1.933 \pm 0.91\%$	83.8	0.94	4.109 ± 0.039
					$\bar{x}_7$	29.1 ± 8.7			$\bar{x}_7$ 4.123 ± 0.025
1	0.7	10.40	1.689	2.210	31.2	$1.879 \pm 1.00\%$	85.0	0.88	3.994 ± 0.042
95-38 (K95-5490); upper pumiceous tuff, 0027/148-156, Kanapoi; $J=0.001177 \pm 0.30\%$; level 6, ANU4/211									
crystal 1	1.3	1.850	2.480	2.015	21.2	$1.935 \pm 0.42\%$	96.0	2.60	4.105 ± 0.021
2	0.8	2.280	4.777	2.032	11.0	$1.942 \pm 0.48\%$	95.6	1.86	4.120 ± 0.023
3	1.3	1.672	2.614	2.031	20.1	$1.957 \pm 0.38\%$	96.4	2.37	4.151 ± 0.020
4	1.4	52.22	2.485	3.492	21.2	$1.923 \pm 0.87\%$	55.1	2.07	4.079 ± 0.037
5	1.3	6.886	2.235	2.163	23.5	$1.934 \pm 0.39\%$	89.4	2.65	4.102 ± 0.020
7	1.9	3.194	1.952	2.059	27.0	$1.939 \pm 0.38\%$	94.2	2.65	4.112 ± 0.020
8	1.0	3.778	2.142	2.078	24.6	$1.941 \pm 0.40\%$	93.4	1.54	4.117 ± 0.021
					$\bar{x}_7$	21.2 ± 5.1			$\bar{x}_7$ 4.112 ± 0.022
6	2.1	1.502	2.342	2.146	22.5	$2.076 \pm 0.36\%$	96.8	3.25	4.404 ± 0.020
95-37 (K94-5385); lower pumiceous tuff, section CSF 94-7/1, 0027/127-098, Kanapoi; $J=0.001176 \pm 0.30\%$; level 4, ANU4/211									
crystal 1	0.9	8.215	2.252	2.206	23.4	$1.936 \pm 0.49\%$	87.8	0.81	4.104 ± 0.024
2	1.8	1.360	2.246	2.041	23.4	$1.974 \pm 0.91\%$	96.7	3.02	4.183 ± 0.018
3	1.5	13.88	3.653	2.404	14.4	$1.968 \pm 0.23\%$	81.9	2.47	4.171 ± 0.016
4	1.2	2.279	2.348	2.074	22.4	$1.980 \pm 0.38\%$	95.4	2.23	4.196 ± 0.020
5	1.3	1.836	2.305	2.048	22.8	$1.966 \pm 0.37\%$	96.0	1.73	4.167 ± 0.020
7	1.2	2.868	0.796	2.066	66.1	$1.952 \pm 0.26\%$	94.5	1.95	4.137 ± 0.017
8	1.1	1.740	0.745	2.060	70.7	$1.980 \pm 0.30\%$	96.1	1.85	4.197 ± 0.018
9	1.3	2.386	5.549	2.073	9.5	$1.979 \pm 0.24\%$	95.4	2.45	4.194 ± 0.016
10	1.2	2.148	2.582	2.042	20.4	$1.952 \pm 0.24\%$	95.6	2.24	4.137 ± 0.016
					$\bar{x}_9$	30.3 ± 22.1			$\bar{x}_9$ 4.165 ± 0.033
6	1.8	8.553	0.486	2.308	108.2	$2.026 \pm 0.24\%$	87.8	3.15	4.295 ± 0.016

Results of single crystal laser fusion $^{40}\text{Ar}/^{39}\text{Ar}$ dating of anorthoclase crystals from a pumice in the Moiti Tuff, east of Allia Bay, and from tuffaceous horizons at Kanapoi, Kenya. The principles of $^{40}\text{Ar}/^{39}\text{Ar}$ dating and the general techniques utilized are given in McDougall and Harrison²². Approximately 20 crystals of feldspar, usually 0.5–1 mm maximum dimension and mass 0.5–2 mg, from each sample were packed in aluminium foil and placed successively in a silica glass tube, interspersed with small packets of sanidine fluence monitor (92-176, separated from the Fish Canyon Tuff, Colorado) of nominal K–Ar age 27.9 Myr^{23,24}. A synthetic potassium silicate glass was placed at either end of the silica glass tube, and the whole package encased in cadmium 0.2 mm thick within an aluminium reactor vessel for irradiation in facility X33 or X34 in HIFAR nuclear reactor, Lucas Heights, New South Wales, for 48 h. The reactor vessel was inverted three times during the irradiation at exactly 6 h intervals, to minimize the effect of neutron flux gradients. Following irradiation, individual alkali feldspar crystals were loaded into wells in a copper sample tray, installed in an ultra high vacuum system and baked overnight at 200 °C. A focused argon-ion laser beam up to 10 W power was used to fuse individual crystals. The gases released from each crystal were purified over SAES getters and then expanded directly into a Fisons VG3600 gas source mass spectrometer operated statically. Argon isotope measurements were made on ^{36}Ar , ^{37}Ar , ^{38}Ar , ^{39}Ar and ^{40}Ar , collecting data through a Daly detector and photomultiplier system. Mass spectrometer control and data acquisition was done using a Macintosh computer and Noble software. Sensitivity of the VG3600 operated at 200 μA trap current and 4.5 kV accelerating potential was about 2.9×10^{-17} mol mV^{-1} for argon on the Daly detector system. Mass discrimination was measured regularly using purified atmospheric argon from a gas pipette; the Daly collector system yielded $^{40}\text{Ar}/^{36}\text{Ar}$ ratios of 289 ± 2 over the course of this work. The irradiation parameter, J , for each unknown was derived by interpolation from the argon isotopic measurements made on gas released from the sanidine fluence monitor crystals (92-176). A minimum of three crystals was measured for each level of fluence monitor in the silica glass tube, with a precision of $\leq 0.3\%$ (standard deviation of the population). The ^{37}Ar and ^{39}Ar were corrected for decay. System blanks and backgrounds in the VG3600 were small to negligible. $\lambda = 5.543 \times 10^{-10} \text{ yr}^{-1}$; $^{40}\text{Ar}^*$ is radiogenic argon, $^{39}\text{Ar}_K$ is K-derived ^{39}Ar . The quoted error for each age includes estimated error in J , the irradiation parameter. ($^{36}\text{Ar}/^{37}\text{Ar}$)_{ca} = 3.49×10^{-4} ; ($^{39}\text{Ar}/^{37}\text{Ar}$)_{ca} = 7.86×10^{-4} ; ($^{40}\text{Ar}/^{39}\text{Ar}$)_K = 0.018 to 0.032, depending on position.

TABLE 3 Results of whole rock dating

Sample number	K wt (%)	Radiogenic ^{40}Ar	$100 \times \text{Rad. } ^{40}\text{Ar}$	Calculated age Myr ± 1 s.d.
		$10^{12} \text{ mol g}^{-1}$	Total ^{40}Ar	
93-1052	0.959, 0.956	5.17	30.7	3.11 ± 0.04
93-1051	0.871, 0.874	5.16	26.0	3.41 ± 0.04

K-Ar age results on whole rock samples of the Kalokwanya Basalt, near Kanapoi, Kenya. $\lambda_e = 0.581 \times 10^{-10} \text{ yr}^{-1}$; $\lambda_\beta = 4.962 \times 10^{-10} \text{ yr}^{-1}$; $^{40}\text{K}/\text{K} = 1.167 \times 10^{-4} \text{ mol mol}^{-1}$. 93-1052: Basalt capping sandstone and conglomerate, air photo coordinates 0025/122-126. 93-1051: Basalt overlying conglomerate, air photo coordinates 0025/125-073.

tions on single crystals. Thus alkali feldspar was separated from clasts in sample 95-37 from the basal claystone in section CSF94-7 (Fig. 2), the lower of the two tuffaceous horizons. From the stratigraphically higher tephra, two separate samples of claystone (95-36, 95-40) from the same locality, near the base of section CSF94-8 (Fig. 2), provided alkali feldspar concentrates derived from the altered clasts. A further feldspar concentrate was prepared from sample 95-38 collected from a correlative horizon located about 1.3 km north northeast of section CSF94-8 (Fig. 2). Results of total fusion ^{40}Ar - ^{39}Ar dating of 8-10 single alkali feldspar crystals from each sample are given in Table 2, the footnotes of which describe the techniques used. As the uncertainty in each age generally is comparable at about 0.5% standard deviation, a simple mean age was calculated for the results from each sample. Any measured age differing by more than two standard deviations from the mean was eliminated as an outlier and a new mean and standard deviation of the population calculated. For each Kanapoi sample, one single crystal result was excluded by this criterion, and shown in Table 2 in italics. In general we have preferred to quote uncertainties in the

pooled ages as the standard deviation of the population rather than of the mean, as the latter yields values that are misleadingly small, especially when uncertainties in the irradiation parameter, J , of 0.3% are taken into account.

Feldspars from the lowermost tephra-bearing horizon, from sample 95-37, yielded a concordant set of measured ages on 9 crystals with a simple mean of 4.165 ± 0.033 Myr, after excluding one older age (Table 2). The weighted mean age is 4.167 ± 0.004 Myr, where the statistic quoted in this case is the standard deviation of the mean.

Single crystal alkali feldspar ages from the three samples (95-36, 95-38, 95-40) from the stratigraphically higher tuffaceous horizon at Kanapoi yield simple mean ages of 4.112 ± 0.022 , 4.123 ± 0.025 and 4.119 ± 0.005 Myr, respectively (Table 2). These mean ages cannot be distinguished from one another at the 95% confidence level, consistent with sampling a homogeneous population of feldspars in each case, as was expected from the stratigraphic position. Thus it is legitimate to combine all 21 accepted single crystal results from this horizon to yield a simple mean age of 4.118 ± 0.019 Myr (weighted mean age is 4.121 ± 0.004 Myr).

Statistical analysis of the ^{40}Ar - ^{39}Ar ages for the two pumiceous horizons from the lowermost stratigraphic unit at Kanapoi shows that they are readily distinguishable at the 95% confidence level; note that the results are in accord with the stratigraphic order.

It is inferred that these alkali feldspars are volcanic in origin, having crystallized from rhyolitic magmas that erupted explosively. The measured ages are considered to record the cooling of the feldspar crystals at the time of eruption. Deposition of the pumiceous material in the Kanapoi sequence is likely to have occurred very soon after explosive eruption.

TABLE 4 Fossil mammals from Kanapoi

Order	Family	Subfamily/tribe	Genus	Species
Macroscelidea	Macroscelididae		<i>Rhynchocyon</i>	sp.
Primates	Lorisidae	Galaginae	<i>Galago</i>	cf. <i>senegalensis</i>
	Cercopithecidae	Colobinae	gen. nov.	sp. nov.
		Cercopithecinae	<i>Parapapio</i>	aff. <i>ado</i>
	Hominidae		<i>Australopithecus anamensis</i>	
Rodentia	Scuiridae			
	Cricetidae		<i>Tatera</i>	sp.
	Muridae			
	Hystriidae		<i>Hystrix</i>	sp.
Carnivora	Mustelidae		<i>Enhydriodon</i>	sp.
	Viverridae		gen. indet.	
	Hyaenidae		<i>Parahyaena</i>	sp.
Proboscidea	Deinotheriidae		<i>Deinotherium bozasi</i>	
	Gomphotheriidae	Anancinae	<i>Anancus kenyensis</i>	
	Elephantidae	Elephantinae	<i>Loxodonta audorora</i>	
Perissodactyla	Equidae		<i>Hipparion</i>	sp.
	Rhinocerotidae	Rhinocerotinae	<i>Ceratotherium praecox</i>	
Artiodactyla	Suidae		<i>Nyanzachoerus kanamensis</i>	
			<i>Nyanzachoerus jaegeri</i>	
	Hippopotamidae		<i>Hexaprotodon</i>	cf. <i>protamphibius</i>
	Giraffidae		<i>Giraffa</i>	aff. <i>jumae</i>
			<i>Giraffa</i>	aff. <i>stillei</i>
	Bovidae	Tragelaphini	<i>Tragelaphus kyaloe</i>	
		Bovini		
		Reduncini	<i>Kobus</i>	sp. nov.
		Hippotragini		
		Alcelaphini	? <i>Damalacra</i>	sp.
		Aepycerotini	<i>Aepyceros</i>	sp.
		Antelopini	<i>Gazella</i>	sp.
		Neotragini	<i>Madoqua</i>	sp.
			<i>Raphiceras</i>	sp.

Within the deltaic interval, a vitric tephra, termed the Kanapoi tuff (Fig. 2), has been correlated with the Suteijun Tuff of the Chemeron Formation in the Baringo Basin¹⁵. Although not dated, this tephra is about 20 m below a geochemical correlative of the Lokochot Tuff (3.5 Myr¹⁶). A younger limit for the deposition of the sedimentary rocks at Kanapoi is given by K–Ar ages of 3.11 ± 0.04 Myr and 3.41 ± 0.04 Myr on whole-rock samples of the Kalokwanya basalt from the scarp northeast of Kanapoi (Fig. 2, Table 3). The measured ages on the basalts are regarded as reliable minima, as they contain a mesostasis, which may not retain radiogenic argon quantitatively. The combined isotopic dating evidence, therefore, indicates that deposition of the Kanapoi sedimentary sequence occurred during the interval from ~ 4.17 Myr to >3.4 Myr ago in the Pliocene.

However, there are good reasons for suggesting that the hominid fossils are derived from a more restricted time interval, based upon palaeogeographic evidence. The fossils come from the lower part of the Kanapoi sequence (Fig. 1), which is correlated with the Lonyumun Lake phase of the Turkana Basin²⁵. The upper fossiliferous zone is in sediments regarded as reflecting local deltaic infill of the lake. Our age measurements from Kanapoi show that the Lonyumun Lake came into existence about 4.2 Myr ago, consistent with data from elsewhere in the Turkana Basin²⁵. The Lonyumun Lake sediments are overlain by the Moiti Tuff, which has an age of 3.9 Myr (see below). Although the Moiti Tuff has not been found at Kanapoi, it occurs overlying the Lonyumun Lake sediments at Nakoret, about 50 km north of Kanapoi, as well as elsewhere in the Turkana Basin²⁵. On this basis we believe that the hominid fossils from Kanapoi are most probably confined to an age interval from 4.2 to 3.9 Myr.

Most of the vertebrate fossils at Kanapoi come from two stratigraphic levels. The lower level is the channel sandstone and overbank mudstone complex associated with the pumiceous tephra dated at 4.17 ± 0.02 and 4.12 ± 0.02 Myr. The upper fossiliferous zone is the distributary channel associated with the Kanapoi tuff (>3.5 Myr). Many of the hominid fossils, including the holotype mandible (KNM-KP 29281) and the maxilla (KNM-KP 29283), are from the lower level, and are thus tightly constrained in age between 4.17 Myr and 4.12 Myr. The tibia (KNM-KP 29285), the distal humerus (KNM-KP 271) and two

mandibular fragments (KNM-KP 29287) are from the upper level, older than 3.5 Myr and younger than 4.1 Myr.

The hominids from Allia Bay derive from beneath or within the Moiti Tuff¹⁷. Single crystal dating of 9 feldspar crystals from a pumice collected from the Moiti Tuff in the Koobi Fora region provided an essentially concordant set of ages with a simple mean of 3.92 ± 0.04 Myr (Table 2) or a weighted mean of 3.94 ± 0.01 Myr. This is younger than our previously published best estimate of $\leq 4.10 \pm 0.07$ Myr¹⁸ based on K–Ar age measurements on bulk alkali feldspar separates from three pumices. White *et al.*¹⁹ reported a ^{40}Ar – ^{39}Ar weighted mean age of 3.89 ± 0.02 Myr for feldspars from tuff VT-1 in the Maka area of Ethiopia. This tuff is regarded as a correlative of the Moiti Tuff on geochemical grounds. The age measurements are consistent with this suggested correlation.

Palaeoecology and fauna

The Kanapoi fossil vertebrates include over 30 mammalian taxa (Table 4), but fish and aquatic reptiles are common. A small *Parapapio*, *Parapapio* cf. *ado*, dominates the cercopithecids; only two colobine specimens were found. The most common carnivore was a precursor of the brown hyaena, cf. *Parahyaena*. The dominant bovid was a medium sized tragelaphine, *Tragelaphus kyalaoe*. Kudus and impalas (in the ratio 12:1, respectively) account for over half of the bovids, with antelopes and alcelaphines making up a further third, and bovines the remainder (J. M. Harris, personal communication).

Early hominids were apparently not restricted to a narrow range of habitats. Dry, possibly open, wooded or bushland conditions are indicated by the Kanapoi mammalian macro- and microfauna, although a wide gallery forest would have almost certainly been present on the large river that brought in the sediments. At Allia Bay there is a fauna that appears to be associated with the large proto-Omo river¹⁷, and a gallery forest is also indicated there by the presence of several large catarrhine species. These sites stand in contrast to Aramis, which is interpreted to have been closed woodland³. At Aramis, aquatic species and large mammals are rare, and colobines make up over 30% of all vertebrate specimens collected. The Laetoli palaeo-environment is believed to have been rather open with grassland, scattered trees and nearby woodland²⁰. A changing mosaic of habitats existed at Hadar, including closed and open woodland, bush and grassland²¹. □

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Modulation of memory fields by dopamine D1 receptors in prefrontal cortex

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Dopamine has been implicated in the cognitive process of working memory but the cellular basis of its action has yet to be revealed. By combining iontophoretic analysis of dopamine receptors with single-cell recording during behaviour, we found that D1 antagonists can selectively potentiate the 'memory fields' of prefrontal neurons which subserve working memory. The precision shown for D1 receptor modulation of mnemonic processing indicates a direct gating of selective excitatory synaptic inputs to prefrontal neurons during cognition.

THE deficits in working memory evident in Parkinson's disease¹ and schizophrenia² have been postulated to arise from a deficiency in the function of prefrontal cortex³. Reduced cortical dopamine function has been implicated in both disorders, and it has been shown that local depletion of dopamine in primate prefrontal cortex impairs working memory⁴. Conversely, raising dopamine levels in schizophrenic patients by amphetamine⁵, or in Parkinson's patients by L-3,4-dihydroxyphenylalanine (L-DOPA)⁶, improves their performance on tests that utilize working memory. Treatment of both disorders has emphasized the significance of the D2 receptor in drug medication⁷. However, experimental studies in non-human primates^{8,9} have implicated the D1 receptor family in the working memory functions of the prefrontal cortex, where these receptors are far more abundant than the D2 receptor¹⁰. Moreover, the dopamine-induced facilitation of NMDA (*N*-methyl-D-aspartate) excitatory amino-acid receptor action that has recently been demonstrated in *ex situ* human cortex¹¹ is probably mediated by D1 receptors¹². To determine the role of different dopamine receptors in working memory function we have examined the action of D1 and D2 antagonists on neurons in primate prefrontal cortex that exhibit 'memory fields'. These neurons are considered to be the cellular basis for working memory because: (1) they increase firing when the monkeys must retain the location of a target during a delay period between target presentation and time of response; (2) different prefrontal neurons encode different target locations; and (3) failure of these neurons to maintain their activity during the delay is invariably associated with errors in behavioural performance^{13,14}. For the purpose of this study, we have developed a simple method for iontophoresing drugs on to neurons in chronic single-unit studies of monkeys performing working memory tasks. This technique has allowed us to isolate and identify the effects of selective dopamine receptor antagonists on the dissociable mnemonic and sensorimotor processes displayed by prefrontal neurons^{13,15} during normal delayed-response performance. We show that the D1 receptor has a specific role in regulating neuronal activity associated with the mnemonic process itself. The importance of this receptor to cognition parallels its significance for psychomotor function as highlighted by recent studies of D1 mutant mice^{16,17}.

Neuronal activity was recorded from the dorsolateral prefrontal cortex in two monkeys (cared for in accordance with federal regulations and approval of the Yale University Animal Care Committee) while they performed an oculomotor delayed-response (ODR) task in which the spatial position of a stimulus had to be remembered and updated on a trial by trial basis¹³. Each neuron was tested for eight peripheral target locations (Fig. 1a) presented randomly over multiple trials in both control and drug conditions. Neuronal activity was classified on the basis of responses time-locked to the cue, delay and/or response periods

within a trial. Concurrent iontophoretic recording used specially designed 'quad-channel' carbon-fibre electrodes containing three barrels for drug ejection.

Specific action of D1 antagonists

Of 132 single units recorded in the caudal principal sulcus and rostral arcuate sulcus, 72 were successfully tested for the effects of at least one selective dopamine receptor antagonist on cell firing during the ODR task; 59 of these cells had classified responses related to one or more periods of the task. Consistent with our previous reports, prefrontal neurons often exhibited spatially tuned 'memory fields'; that is, they responded maximally during the delay period for targets in one or a few adjacent target locations, and not above baseline for any other location ($n = 34$ cells). Iontophoresis of SCH 39166 (Schering-Plough), a selective D1 antagonist^{18,19}, clearly and consistently enhanced these memory fields when applied at low ejection currents (20–50 nA). For example, as shown in Fig. 1b, neuron W54 displayed an increase in activity for target position 2 (top left), but not for other target positions, including position 7 (top right). Delay activity (and the phasic cue and response period activity occurring in conjunction with it) was enhanced by SCH 39166 for target 2 (Fig. 1b, left), but not for any target locations outside the memory field. Indeed, SCH 39166 accentuated the reduction in activity during the delay period which often occurs on trials with targets in the location opposite to the memory field (an 'opponent memory field'; Fig. 1b, right). Finally, the enhanced activation for target 2 during the delay could be reversed by iontophoresis of the partial D1 agonist SKF 38393 onto the cell, and this reversal was also selective to the neuron's memory field (Fig. 1b). The enhancement effects were replicated in 11 out of 12 neurons. Two of these cells displayed only inhibitory memory fields¹³. As for opponent memory fields, SCH 39166 enhanced inhibitory responses during the delay period selectively in both cells tested (data not shown). Together these findings provide evidence of a unique dopaminergic modulation that can be extraordinarily precise, affecting a specific component of the neuron's afferent (excitatory and inhibitory) input without affecting its general excitability. Similar results were obtained with the less-selective D1 antagonists, SCH 23390 (5 of 7 cells), NNC 01-0756 (8 of 11 cells) and A 60924 (4 of 4 cells), although these drugs tended to accentuate the memory field by reducing the background activity of the cell rather than directly enhancing its delay activity.

Dose dependency of D1 receptor function

That D1 receptor blockade could enhance the neuronal signal in the delay period is suggestive of a memory-enhancing action at the doses used. However, previous studies with intracortical or systemic injection of drugs have suggested that D1 blockade

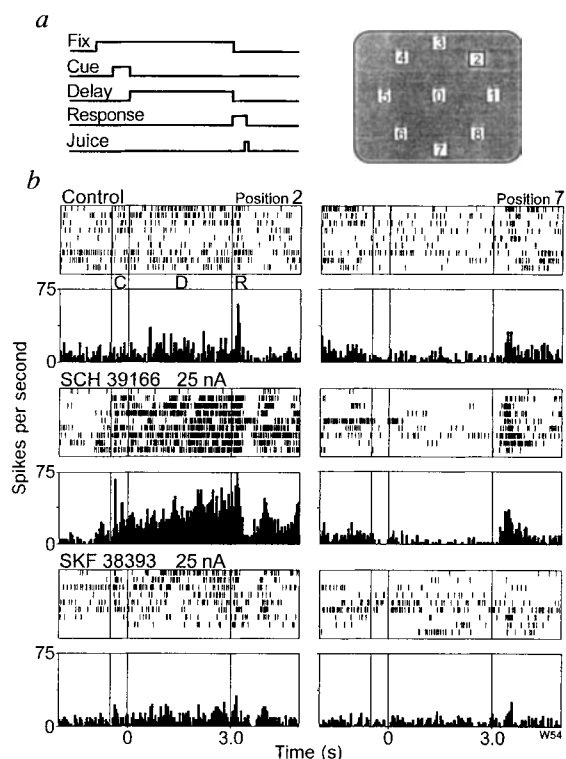


FIG. 1 *a*, Left, schematic sequence of events in each phase of the ODR task (aligned in time with rasters and histograms shown immediately below); right, depiction of the 8 positions of the target (plus the central fixation position, 0) to be remembered for guidance of oculomotor response. *b*, Effect of SCH 39166 on response of neuron W54 (raster-gram above, histogram below; bin, 50 ms) for a target (position 2) in the memory field (left) and for a target (position 7) in nearly the opposite location in space (right). Top two rows, control recording showing significant but weak delay activity. Middle two rows, SCH 39166 (25 nA) produces dramatic enhancement of activity during the delay period when the target is in the memory field but produces an inhibition of activity when the target is in position 7. Bottom two rows, SKF 38393 reverses the effect of SCH 39166 and reduces delay activity to 'background' level. C, cue period; D, delay period; R, response period.

METHODS. In the ODR task a monkey is required to fixate on a small central stimulus (approximately subtending 1° of visual space) for 0.5 s, after which a peripheral cue stimulus (13° eccentric, 45° separation) appears for 0.5 s in one of eight positions (see *a*; position randomly allocated for each trial). The monkey has to maintain its central fixation for a further 3 s during the delay period. After this time, the fixation stimulus is removed and the monkey has to make a rapid saccade to, and fixate upon, the location where the cue stimulus had been shown. Each neuron was tested for 8 peripheral target locations (as shown in *a*) presented randomly over multiple trials in both control and drug conditions. Analysis of variance was used to compare neuronal responses with regard to: (1) different periods of the task (including cue, delay, response and inter-trial interval); (2) different drug conditions; and (3) different target locations. The source of significant variance was then ascertained by Tukey's ω -procedure. All delay responses and their spatial tuning, and all drug effects, were based on significant comparisons ($P < 0.05$). Iontophoresis was started after sufficient numbers of trials (usually 9 or more) had been collected for each target position in the task under the control condition. Data were taken after drug had been applied for at least 10 s. In most cases the neuron was recorded in recovery from the drug (≤ 45 min) to check that activity returned to approximate control levels. While drugs were not being tested, a retaining current of 1–2 nA was applied to each drug channel periodically. Iontophoretic electrodes were constructed from quad-sectioned glass tubing (Clark Electromedical, Pangbourne, U.K.) using 33- μ m carbon fibre (Textron Inc., Lowell, MA)⁴⁴. The glass was flame polished at the top end, and the assembly was 'pulled' using a computer-driven device to produce long electrode shanks (90–100 mm) with fine tips (~ 35 μ m) which were then silver plated for low-noise recording⁴⁵. The electrodes were then loaded with drugs at a concentration of 5–10 mM, pH 3.5–4.0.

is detrimental to working memory performance^{8,9}. The explanation for these discrepancies may be related to dosage. To determine whether the effect of the D1 antagonists on neuronal activity is entirely dose-dependent, we tested the effect at high doses (>50 nA ejection current) on 5 neurons with memory fields. In sharp contrast to the selective enhancement effect of low doses described above, high ejection currents inhibited firing nonspecifically, affecting not only the memory field of the cells but also neuronal activity throughout all periods of the task regardless of target location (cell W56, Fig. 2). These results suggest that excessive D1 receptor blockade may render prefrontal cells unresponsive to their normal functional inputs, possibly by means of indirect mechanisms involving inhibitory local circuits²⁰. This may explain the previously observed deficits when D1 antagonists are injected either locally or systemically at particular doses^{8,9}, and also accounts for earlier reports of inhibition of delay-period activity produced by non-selective dopamine antagonists²¹.

Mnemonic versus sensorimotor activity

We next examined the effects of SCH 39166 on another class of prefrontal neuron, those with activity specifically related to the cue or response periods of the task, and not associated with the delay period. Doses of SCH 39166 that enhanced delay activity in other neurons produced no increase in cue- or response-related phasic activity in 5 out of 6 cases (Fig. 3). Similarly, SCH 23390, NNC 01-0756 and A 60924 never increased activity during the cue or response period but actually reduced activity in these neurons (18 of 19 cells). This result shows that neurons with memory fields may have a particularly high density of D1 receptors. The dissociation observed in the effects of a D1 antagonist on mnemonic and sensorimotor processing in prefrontal cortex concurs with the well-established sparing of sensory-guided performance in various tasks after lesions of prefrontal cortex^{22,23}, local dopamine depletion⁴, or intracortical injection of D1 antagonists into the same area⁸.

D1 and D2 receptor function

We have determined that the modulation of delay period activation by the D1 antagonist was pharmacologically specific.

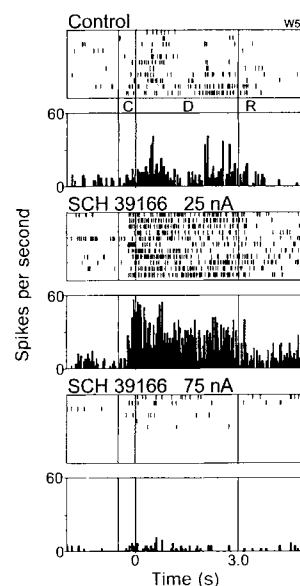


FIG. 2 Dose-dependent effect of SCH 39166 on activity of neuron W56. Top two rows, control recording showing significant delay period activity. Middle two rows, SCH 39166 (25 nA) induces dramatic enhancement of delay activity. Bottom two rows, SCH 39166 at a high dose (75 nA) abolishes activity during the delay period as well as other periods of the task.

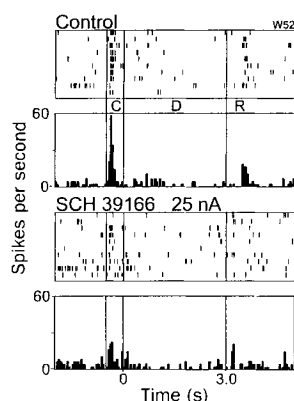


FIG. 3 Effect of SCH 39166 on cue and response activity in a neuron (W52) with no memory field. Top two rows, control; bottom two rows, SCH 39166. Both the cue and response period activity are actually attenuated in this neuron at an ejection current (25 nA) which normally enhanced delay activity in other cells.

Iontophoretic application of raclopride (Astra), a D2/D3 antagonist²⁴, was examined in 14 of the 72 functionally characterized neurons (13 of which were also tested with D1 antagonists). In 10 cases, at doses ranging from 10 to 50 nA, raclopride produced only a generalized inhibition; that is, cell activity was diminished in all phases of the task including the delay period, as shown by cell W85 in Fig. 4a. This inhibitory effect of a D2 antagonist on functionally characterized neurons would not have been expected from previous studies on the effects of intracortical injection of this drug⁸ or iontophoresis of sulpiride²¹. When we compared the effects of SCH 39166 and raclopride on delay period activity (see Fig. 4b) it was clear that, whereas SCH 39166 enhanced memory fields of prefrontal neurons, raclopride had only the general action of inhibition across all neurons tested with the drug. If D2 receptor stimulation has any role in the mnemonic component of the delayed-response task, it must be one that is less integral to the mechanisms involved than the D1 receptor. This finding is consistent with autoradiographic evidence that the D2 receptor is very weakly represented (approximately 2–3 fmol per mg tissue) in prefrontal cortex, whereas the D1 receptor is present in approximately ten times greater concentrations¹⁰.

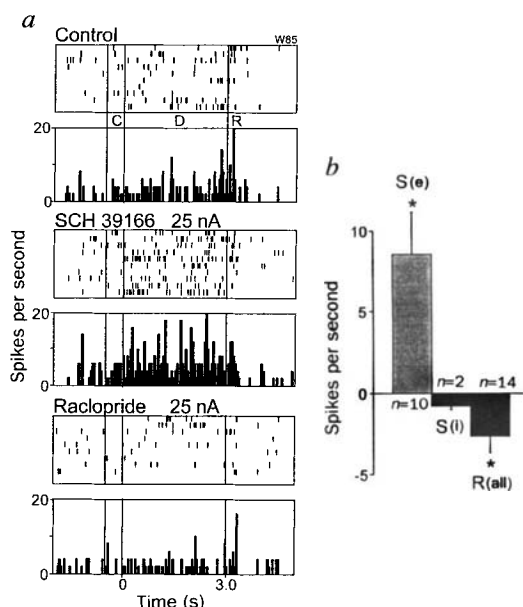
FIG. 4 Comparison of effects of SCH 39166 and raclopride on delay activity. a, Response of cell W85 to both drugs (data from trials with optimum target location). Top two rows, control recording showing activation in the delay period. Middle two rows, SCH 39166 (25 nA) boosts delay activity. Bottom two rows, raclopride (25 nA) attenuates delay activity in comparison with control. b, Bar chart of delay activity under both drug conditions relative to control. SCH 39166 induces a significant elevation of delay period activity ($P < 0.01$, two-tailed *t*-test) overall, in 10 cells with excitatory memory fields (S(e)), and a further inhibition of activity in the two cells with inhibitory memory fields (S(i)). In contrast, raclopride produces a significant diminution of activity during the delay period ($P < 0.05$, two-tailed *t*-test) when examined over all 14 cells tested (R(all)).

Anatomical basis of D1 specificity

These findings reveal a highly specific contribution of the D1 receptor to cognitive function by direct modulation of the memory fields of prefrontal neurons, the presumed neuronal basis of working memory. Taken together, they show that there may be an optimal level of D1 receptor activation for the best resolution of spatial mnemonic processing in prefrontal neurons. The enhancement of activity was found to be highly selective to prefrontal neurons with memory fields. Delay activity for all other target locations outside the fields was not enhanced, and the cue and/or response period activity found in other prefrontal cells was usually attenuated. This strongly suggests that, in the primate, D1 receptors are selectively distributed on prefrontal neurons in a way which corresponds to those excitatory inputs responsible for generating delay period activity. Thus, by means of D1 receptors, dopamine can selectively regulate the mnemonic component of prefrontal cortical function without affecting normal sensorimotor processing in this area of the brain.

We cannot from our study isolate which member(s) of the family of recently cloned D1 receptors is involved^{25,26}. It has been shown that D1 receptors are located preferentially on the distal dendrites and spines of pyramidal cells, but D5 receptors can also be observed on spines as well as dendritic shafts^{27,28}. The localization of the D1 receptor to the spines of prefrontal cortical neurons which also possess asymmetric synapses associated with glutamatergic input²⁹ appears to provide a refined neural substrate for the highly constrained, spatially selective effects of D1 antagonists on cell firing. This anatomical precision of synaptic circuitry within prefrontal cortex³⁰ supports the extraordinarily specific function of dopamine D1 receptors shown here.

These results show that blockade of D2 receptors with raclopride failed to enhance memory fields, instead producing a non-selective reduction in neuronal activity. This suggests that working memory might actually be impaired by D2 receptor blockade in the course of conventional neuroleptic treatment and could possibly be improved by D2 agonists, as has been found in studies of both human³¹ and non-human primates³². In rodents, however, D2 agonists have been reported to impair working memory, suggesting a possible species difference in the dopaminergic modulation of cognition³³. In accord with this finding, it has proven difficult to demonstrate D1 receptor modulation of cortical neuronal activity in anaesthetized rats, but effects of D2 receptors are prominent³⁴. This may be due to dopamine



innervation of rodent prefrontal cortex being substantially different from that of primates³⁵, and much less associated with dendritic spines^{36,37}. However, there are several possible differences between the studies in rodents and primates which could account for the above drug effects, and further investigation is needed.

Mechanism of memory field enhancement

The enhancement of neuronal activity by D1 antagonists and its reversal by SKF 38393, a D1 agonist, indicates that the normal action of dopamine is to constrain neuronal activation during performance of a working memory task. Such an inhibitory role for dopamine is fully in accord with numerous previous physiological studies³⁸. A known mechanism of inhibition by D1 action is the attenuation of a slow inward sodium current which normally supports activation of the cell by excitatory inputs³⁹. The specificity of D1 receptor action for particular inputs in the present study, in combination with ultrastructural analysis²⁹, indicates that blocking the D1 receptor may simply disinhibit specific excitatory input to the same cells. This finding is in accord with the demonstrated lack of normal inhibition of neuronal activity by iontophoresis of dopamine in D1 mutant mice¹⁷.

That iontophoresis of D1 antagonists at levels below 30 nA enhanced delay period activity selectively, whereas high levels of the antagonist (>50 nA) practically abolished the activity of these neurons, leads us to conclude that there is an optimal level of D1 receptor occupancy for the generation of memory fields. Presumably, cortical dopamine levels are elevated towards the high end of the normal range under task conditions which require a high level of motivation. Under such conditions, blocking a proportion of D1 receptors prevents their supraoptimal activation. These considerations emphasize how finely tuned is the dopaminergic regulation of cortical processing, and may help to explain the vulnerability of these processes to dopamine dysfunction in conditions of stress^{40,41}, ageing^{9,32}, drug use⁴² and disease^{5,6}. This raises the question of whether the failure of neuroleptic drugs to alleviate the cognitive deficits of schizophrenia relates to an excessive blockade of cortical D1 receptors in these patients. Indeed, schizophrenic patients, with possibly reduced cortical dopamine as a result of neuroleptic

therapy, show improved working memory performance when given amphetamine⁵. The enhancement of neuronal activity by limited blockade of D1 receptors further suggests that it may be possible to improve memory guided performance by appropriate doses of D1 antagonists when given systematically, and behavioural studies in progress are designed to address this issue.

Modulation of memory fields

Although iontophoretic application of drugs onto a single cell was highly effective in altering its activity, such microejection of drugs is incapable of altering performance on the behavioural task, and indeed we did not observe any such effect. Iontophoresis applies very small quantities of a drug to a highly localized area in the vicinity of the recording electrode. To influence behaviour it would be necessary to inject the drug at sufficiently high concentrations either locally into the cortex or systematically. When D1 antagonists have been delivered by intracortical pressure injection⁸ or systemic injection⁹, the result has been a deterioration in delayed-response performance. We have shown that iontophoresis of SCH 39166 at greater than 50 nA onto prefrontal cells can dramatically reduce their activity in the oculomotor delayed-response task. Because failure of a neuron to maintain its delay period activity is invariably associated with errors in behavioural performance¹³, we can now interpret the behavioural impairments reported in previous studies as resulting from excessive D1 receptor blockade and a highly suboptimal level of occupancy of this receptor by the endogenous agonist. At the neuronal level this would be manifested as an abolition of prefrontal neuronal activity, including memory fields, which could result from the loss of D1 receptor facilitation of excitatory NMDA receptor input¹². If our findings also apply to other cortical areas, they may provide insight into the mechanisms underlying the dose-dependent loss of self-stimulation in medial prefrontal cortex of the rat after local injections of a D1 antagonist⁴³. Moreover, the effects of excessive D1 receptor blockade may have parallels with the effects of complete absence of this receptor, which is open to study in D1 mutant mice¹⁷. The facilitatory and inhibitory mechanisms of cortical neuro-modulation proposed here form a basis for study of the necessary interactions between the D1 receptor and excitatory amino-acid receptors in the dopaminergic modulation of cortical information processing underlying cognition. □

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Radiative acceleration of gas in quasars

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QUASARS can radiate up to a thousand times the energy of the entire Galaxy, yet this energy is generated in a small region approximately one light day across. (By comparison, the diameter of the Milky Way is about 100,000 light years.) Because of the high energy density in this region, it has often been suggested that radiation pressure might play an important dynamical role in quasars¹⁻³. Here we show that radiative acceleration can readily explain a prominent feature observed in the spectra of several broad-absorption-line (BAL) quasars. The broad absorption lines are themselves attributed to matter flowing towards the observer with velocities approaching one-tenth of the speed of light^{4,5}, and our results suggest that radiative acceleration is the dominant driving mechanism in these outflows. As most quasars are believed to have BAL outflows⁶ (although they are seen in only about 10% of them because of viewing angle^{7,8}), radiative acceleration is likely to be an important dynamical process in quasars in general.

In recent years, statistical evidence has been found for the existence of a double trough in the C iv 1,549 Å BAL^{9,6,10}. We have constructed a generic dynamical model¹¹, based on radiative acceleration, which can explain the appearance of such a feature. We use standard BAL-region parameters: a distance of ~ 1 pc from the central source, a quasar luminosity of 10^{46} erg s⁻¹ and a number density of the absorbing gas of $\sim 10^8$ cm⁻³. We note that the location of the BAL region with respect to the central source is very poorly constrained, but is inferred to be beyond the broad-emission-line (BEL) region which is situated at ~ 0.1 pc for a typical bright quasar¹².

The flow accelerates radially as it absorbs the photons' momentum in a relatively small number of resonance-line transitions. If the flux incident upon the absorbing ions (corrected for spherical dilution) and the ionization equilibrium in the outflowing gas both stay constant, pure radiative acceleration predicts constant optical depth as a function of velocity¹³. However, the flux seen by the ions does not stay constant. The strongest modulation is seen by the N v ions. After being accelerated to several thousand kilometres per second, their 1,240-Å transition samples (as a result of the Doppler shift) an increasing radiation flux due to the red wing of the Lyman- α 1,215 Å BEL (which is normally the strongest BEL in quasars). The total radiative acceleration increases and the absorption trough becomes shallower. This trend continues until the N v ions accelerate to $\sim 5,900$ km s⁻¹, which corresponds to the separation between the N v and Ly α rest wavelengths. From that point on, the 1,240-Å transition samples a decreasing flux due to the blue wing of the Ly α BEL, and the acceleration drops, causing an increase in absorption. Because the N v ions share their acceleration with all the other ion species in the flow, the changes in the absorption level will be mimicked in the other BALs. In particular, under favourable conditions (see below) this behaviour will be observed in the C iv BAL as the 'ghost' of Ly α at $-5,900$ km s⁻¹. This effect is expected only if radiative acceleration via resonance line scattering is the dominant driving force in BAL outflows.

The above scheme not only explains the creation of a hump in the C iv BAL at $-5,900$ km s⁻¹, but also constrains the shape and width of the hump to qualitatively resemble those of the Ly α BEL. For example, box-shaped hump centred around $-5,900$ km s⁻¹ cannot be explained by our model as we never observe box-shaped BELs. Because the definition of the hump is more restrictive than that of the double-trough phenomenon^{10,11}, we expect the objects that show the 'ghost' signature to be a subset of the double-trough sample.

Even when the main driving mechanism in all BAL outflows is radiative acceleration, only a few objects are expected to show a clear signature of a Ly α 'ghost' in their spectrum. This is because, in order to exhibit the signature, the dynamical model requires that the spectrum would have all of the following properties¹¹: (1) significant absorption in the range $-3,000$ to $-9,000$ km s⁻¹; (2) a very strong Ly α BEL; (3) relatively narrow BELs, in particular Ly α ; (4) strong absorption in both the C iv and N v BALs (however the optical depth in C iv cannot be

FIG. 1 Data and model fits for quasar 1336+1335 plotted as flux (normalized to the continuum level) versus velocity relative to the quasar rest frame. Solid line, histogram showing the data for the C iv region where zero velocity corresponds to 1,549 Å. Dashed line, histogram showing the N v–Ly α data where zero velocity corresponds to the rest wavelength of the N v line (1,240 Å). Dotted lines, model fits to these spectral regions. The same dynamical model reproduces simultaneously the hump in the C iv BAL (at $\sim -5,900$ km s⁻¹) and the partially absorbed Ly α BEL which appears in the N v BAL. The beginning and end of the overall troughs do not have a strong relation to radiative acceleration. Therefore, we ignore the additional features that occur at high velocities (for example the additional trough in the spectra of 1336+1335 and 1314+0116).

METHODS. The models are an improved version of the dynamics we used in ref. 11, including: the doublet structure of the different lines (see Fig. 2 legend); BALs with different optical-depth ratios (as seen in the data); a small non-radiative acceleration component¹³; a gradual introduction of the flow into the line of sight (using a simple linear introduction model); and changes in the ionization equilibrium across the velocity interval (see Fig. 3 legend). Finally, the unabsorbed emission in the region of each line was modelled (see Fig. 2 legend). A complete discussion of the model will be published elsewhere (N.A., manuscript in preparation).

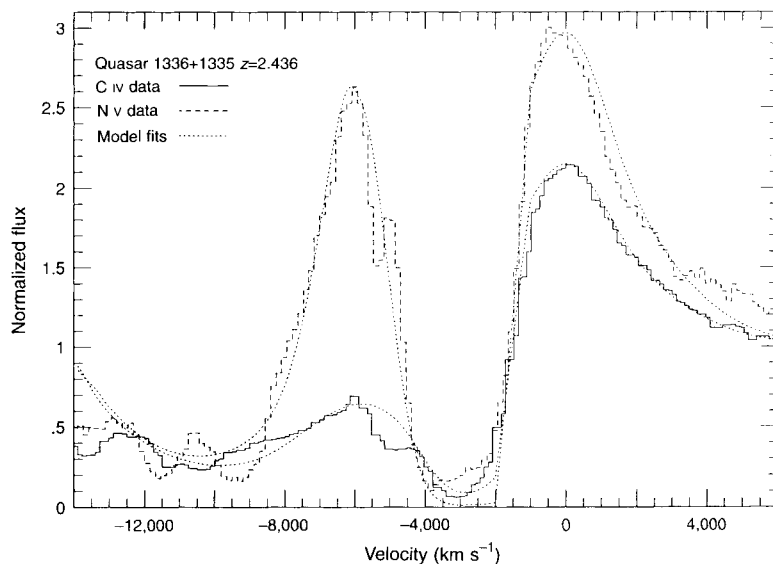
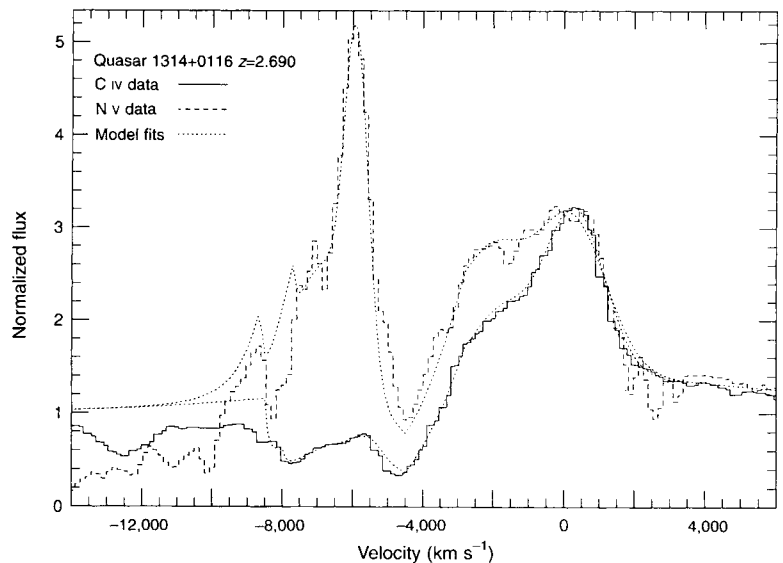


FIG. 2 As Fig. 1, for quasar 1314+0116. The inclusion of the doublet parameters for the BALs in the spectrum of object gave a significant improvement in the fit. The position of the peak of the C iv hump and its asymmetry were fitted much better than in the case where we treated the lines as singlets. This is encouraging because the doublet parameters are not adjustable, and especially as the BELs in this object are the narrowest of those we modelled. The spectrum of this object also shows why it is difficult to model the intrinsic BELs. The C iv BEL shows strong blue asymmetry which we can see only because the absorption starts at $\sim 3,000 \text{ km s}^{-1}$. Furthermore, the asymmetry in the N v BEL looks stronger than that of the C iv BEL. Therefore, the blue side of C iv BEL cannot be modelled by the red side, and the blue side of the N v BEL cannot be modelled by the blue side of C iv. Thus we used the red wing of the C iv BEL as a first approximation, but made substantial deviations from it when fitting the N v and especially the Ly α BELs.



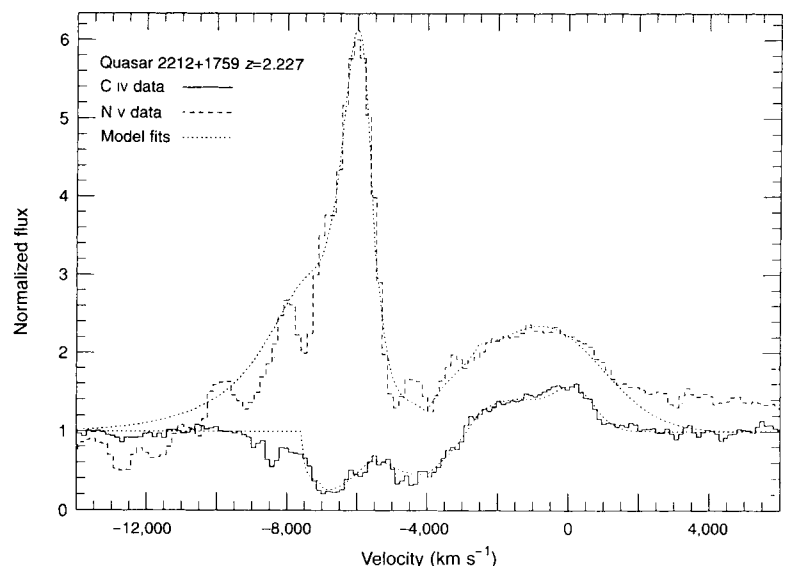
much larger than 3); and (5) a steep decline in the intrinsic continuum at wavelengths below Ly α (more precisely, a strongly reduced flux level between 200 and 1,000 Å, as compared to the spectral region at wavelengths above Ly α). The last requirement stems from the need to suppress the acceleration contribution that arises from the substantial number of resonance lines in that spectral region. We can now use these criteria to predict which BAL quasars out of a given sample should show a clear signature of a Ly α 'ghost' in their spectrum, and then compare our prediction to the observations. For self-consistency we note that by using a steeply declining spectrum and the assumed physical parameters for the BAL region, momentum absorption in three resonance lines can radiatively accelerate the flow to velocity in excess of 20,000 km s $^{-1}$ (ref. 11).

We have analysed a well studied sample of 72 BAL quasars¹⁰, of which only half satisfy the first condition. The second condition is hard to quantify because the Ly α BEL is at least partially absorbed by N v in all the objects that satisfy the first condition. Our criteria for whether an object has a strong Ly α BEL are: either the observed peak of Ly α is at least 300% above the continuum, or the peaks of C iv and N v BELs are both at least 100% above the continuum. (The latter criterion is hardly ideal, but observations of non-BAL quasars show that the line strengths

roughly scale together.) The third condition is met if the full width of the line (as determined by the unabsorbed wing of C iv), at half the maximum intensity (FWHM), is less than 3,500 km s $^{-1}$. This value is based on model runs which show that when the FWHM is larger than $\sim 3,500 \text{ km s}^{-1}$, the N v and Ly α BELs blend enough so that the spectral signature is strongly reduced. The fourth condition is met if, at the point of strongest absorption, it seems that at least half of the incident flux is absorbed in both the C iv and N v BALs. Finally, a decline in the continuum at wavelengths below Ly α cannot be ascertained easily in most objects owing to insufficient spectral coverage at short wavelengths. We can obtain suggestive information from the strength of the He II 1,640 Å BEL; the amount of flux in this line is probably correlated with the flux level at around 200 Å (ref. 14), and a strong line suggests that the intrinsic continuum does not drop sharply in the range 1,000–200 Å.

The first four constraints are satisfied by 8 of the 72 objects in the sample. Four of these (quasars 0029+0017, 1333+2840, 1413+1143 and 2225-0534) either do not show a strong decline in continuum level at wavelengths below Ly α , or show a relatively strong He II BEL. Thus, we do not expect these objects to show a strong signature associated with the radiative acceleration scheme described above. We find that these four objects do

FIG. 3 As Fig. 1, for quasar 2212-1759. In the objects 1336+1335 and 2212-1759, the data from the Si iv 1,397-Å BAL show that there is a significant change in the ionization equilibrium across the velocity interval we model. The ionization equilibrium shifts toward lower ion species with increasing velocity. In particular the Si iv BAL is deeper in the second trough while C iv (and O vi in 1336+1335) becomes shallower. It is not possible to extract the ionization as a function of velocity from the data. However, inserting the observed trend into the model improved the fit. This is encouraging, because *a priori* there is no reason that the observed trend should improve the fit. It is especially helpful in 2212-1759 where the small shift in the location of the C iv hump relative to the Ly α peak is naturally explained by a strong drop in the relative abundance of N v in the range 5,000–7,000 km s $^{-1}$.



not show such a signature, except for 1333+2840 which might have a weak one. Three of the other four objects show a very weak (if any) He II BEL, and two of them show a marked decline of the continuum at wavelengths below Ly α (the third does not have the necessary spectral coverage). These three (1314+0116, 1336+1335 and 2212-1759) all show a strong radiative acceleration signature (Figs 1-3). The last object (1243+0121) is a borderline case which shows a drop in the continuum, but also a moderate He II BEL. Its C IV BAL shows what might be a signature of radiative acceleration, but it is not as persuasive as those of the three modelled. The quasar 1120+019 (not in the sample of 72) also meet all the above constraints, and indeed shows a 'ghost' signature (evident in its O VI 1,035-Å BAL; the spectral region of the C IV BAL was not observed¹⁵).

It is clear that when all the above physical and observational conditions for a radiative-acceleration signature are met, such a signature is indeed observed. Furthermore, the signature is strongly reduced (and in most objects cannot be detected) when even one of these conditions is not satisfied. This correlation is strong evidence that the observed feature is the result of radiative acceleration.

We can estimate the probability that the observed feature occurs randomly in the four objects that we have modelled (including 1120+019). The position of minimum optical depth in the hump is $<300 \text{ km s}^{-1}$ from its expected position in the four objects that show a clear signature. In addition, the dynamical model requires the minimum optical depth of the hump to be ≥ 0.2 (this condition is satisfied for the four ghost objects). Using the sample of 72 BAL quasars we find that seven of them satisfy these two criteria. However, we should not take the individual probability to be 7/72, because only 37 of the objects show appreciable absorption in the appropriate velocity range and our first selection criterion bias us toward that group. Therefore, the probability that a given object has a hump that satisfies the position and optical depth criteria is 7/37. The probability that four out of four objects will satisfy these requirements is therefore $\sim 10^{-3}$. Because the hump has to resemble roughly the Ly α BEL the probability of chance occurrence is reduced drastically, but this reduction is not easily quantifiable. We caution that this kind of an *a posteriori* probability analysis may have unintentional biases that can decrease the significance of the probability result. Also, we note that the process for choosing the objects that satisfy our fifth criterion is partially subjective. Another possible caveat is that by selecting objects with strong observed Ly α emission (as evidence for an intrinsically strong Ly α BEL) combined with significant absorption in N V and C IV in the range $-3,000$ to $-9,000 \text{ km s}^{-1}$, we may bias the selection toward BALs with intrinsic low optical depth at $\sim -5,900 \text{ km s}^{-1}$. However, we try to compensate for this bias by the second criterion we use to define an object with a strong intrinsic Ly α BEL (strong N V and C IV BELs), which should not have this bias.

Our confidence that the Ly α 'ghost' is evidence for the dominance of radiative acceleration in driving BAL outflows is thus based on the following: the appearance of a clear signature when the dynamical conditions are favourable; its absence when the conditions are unfavourable; the small probability of chance occurrence; and our ability to model both the hump in C IV and the N V-Ly α region with the same dynamical model for individual objects, provided that radiative acceleration is the dominant driving force. We predict that these characteristics will be evident in new samples of BAL quasars, and that the 'ghost' of Ly α will appear in the spectra of a few out of every hundred BAL quasars. This feature should show up as a hump near rest wavelength 1,520 Å in the C IV BAL. $\square$

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Template switching between PNA and RNA oligonucleotides

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THE origin of the RNA world¹ is not easily understood, as effective prebiotic syntheses of the components of RNA, the β -ribofurano-side-5'-phosphates, are hard to envisage². Recognition of this difficulty has led to the proposal^{1,3} that other genetic systems, the components of which are more easily formed, may have preceded RNA. This raises the question of how transitions between one genetic system and another could occur. Peptide nucleic acid (PNA) resembles RNA in its ability to form double-helical complexes stabilized by Watson-Crick hydrogen bonding between adenine and thymine and between cytosine and guanine⁴⁻⁶, but has a backbone that is held together by amide rather than by phosphodiester bonds. Oligonucleotides based on RNA are known to act as templates that catalyse the non-enzymatic synthesis of their complements from activated mononucleotides⁷⁻⁹, we now show that RNA oligonucleotides facilitate the synthesis of complementary PNA strands and vice versa. This suggests that a transition between different genetic systems can occur without loss of information.

The synthesis of oligo(G)s on oligo(C) templates using guanosine 5'-phosphoro(2-methyl)imidazole (2-MeImpG; Fig. 1a) as substrate has been described in detail^{10,11}. We began by comparing the deoxynucleotide 10-mer dC₁₀ and PNA-C₁₀ (Fig. 1b, II) as templates for the oligomerization of 2-MeImpG (Fig. 1c, I). The concentrations of reagents, the temperature and the pH were chosen to facilitate comparison with earlier publications⁷. The results are presented in Fig. 2. Both dC₁₀ (Fig. 2b) and PNA-C₁₀ (Fig. 2c, d) are effective templates for the oligomerization of 2-MeImpG. In both cases the yield of oligomeric products is greatly enhanced relative to that obtained in a template-independent reaction (Fig. 2a). However, dC₁₀ is a much more efficient template than PNA-C₁₀, as can be seen by comparing Fig. 2b with Fig. 2c.

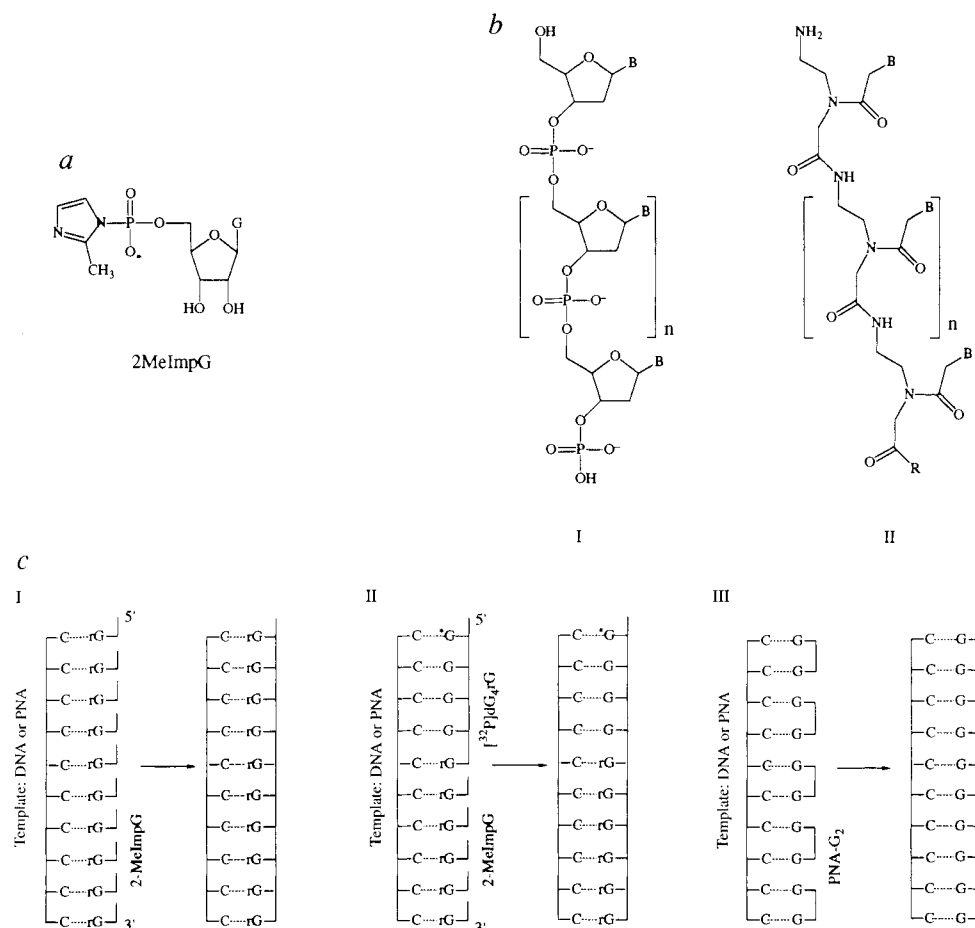
A closer examination of the elution profiles shown in Fig. 2 reveals many details concerning the regiospecificity of the reaction. The series of large labelled peaks observed when the template is dC₁₀ (Fig. 2b) are known to correspond to all 3'-5'-linked oligomers^{10,11}. Co-injection with the products obtained

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FIG. 1 *a*, The structure of guanosine 5'-phosphoro(2-methyl)imidazole (2-MelmpG). *b*, Chemical structures of DNA (I) and PNA (II), where B corresponds to a nucleobase. The PNA-C₁₀ template used in these experiments is derivatized with lysine amide (R is Lys-NH₂) on its carboxyl end⁵. *c*, Schematic representation of template-directed synthesis with I, template C₁₀ (DNA or PNA) and substrate 2-MelmpG; II, template C₁₀ (DNA or PNA), primer [³²P]-dG₄rG and substrate 2-MelmpG; III, template C₁₀ (DNA or PNA) and substrate PNA-G₂. The schemes show only one reaction product, the full-length oligomer. A complex mixture of all possible intermediates up to the full-length oligomer is obtained in our experiments.



with a PNA template established that the labelled peaks in Fig. 2*c, d* also correspond to all 3'-5'-linked oligomers. When dC₁₀ is used as a template, the dimer and trimer are mainly 3'-5'-linked. They are extended to form longer oligomers that are almost exclusively 3'-5'-linked (Fig. 2*b*). With PNA as template the initial synthesis of dimers and trimers yields a complex mixture of products; the 3'-5'-linked isomers are not the major components. However, these and only these 3'-5'-linked oligomers are extended efficiently, and yield longer exclusively 3'-5'-linked products (Fig. 2*c, d*).

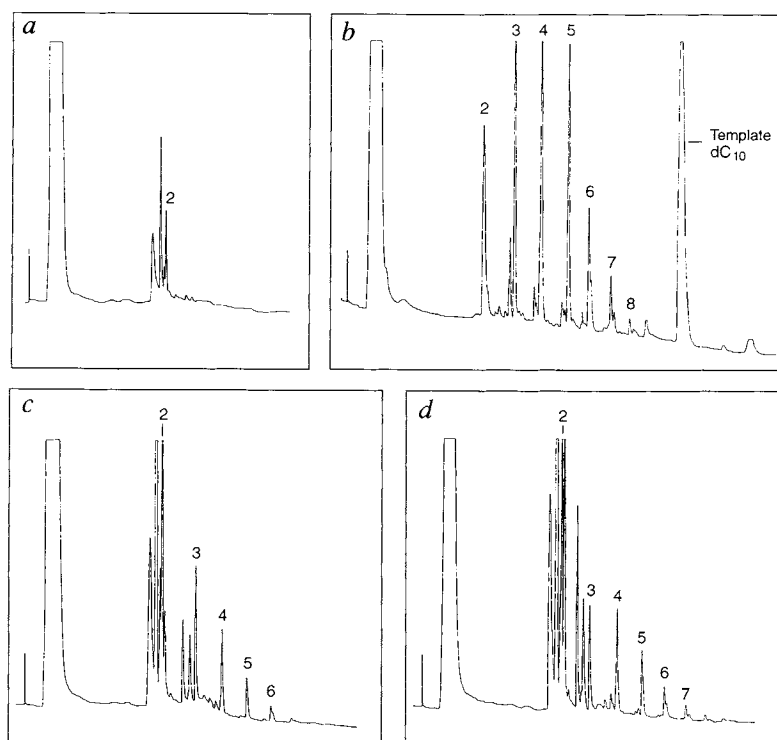
These observations are most plausibly explained if we suppose that a 3'-5'-linked oligonucleotide primer at least 3 bases long is needed to establish an initial double-helical segment with PNA that is isostructural with a DNA:RNA double helix. Once this segment is formed, elongation proceeds efficiently in the same way as with equivalent DNA:RNA helices. The complexes formed by PNA with monomers and dimers of G have a different or less well-defined structure. This leads to the production of short oligomers with 2'-5'-phosphodiester bonds, pyrophosphate-linked oligomers, and possibly cyclic oligomers. These various oligomers form complexes with PNA that are not isostructural with DNA:RNA helices, and consequently they are not elongated regiospecifically. A detailed interpretation of the data presented in Fig. 2*c, d*, provides much evidence for the accumulation of short chain-terminated oligomers at later times in the PNA-catalysed reaction. It is clear, for example, that the 2'-5'-linked trimer accumulates at later times, while the 3'-5'-linked trimer is elongated.

These arguments suggest that the extension of a preformed oligonucleotide primer should proceed efficiently and regio-

specifically on a PNA template. Figure 3 shows the results of an experiment in which a [³²P]-labelled dG₄rG primer is extended by reaction with 2-MelmpG on a dC₁₀ or a PNA-C₁₀ template (Fig. 1*c, II*). It is clear from Fig. 3 that dC₁₀ (lanes 8-10) is only slightly more efficient than PNA-C₁₀ (lanes 5-7) as a template for the primer-extension reaction. The regiospecificity of the reaction was confirmed by hydrolysis of the products of the reaction with ribonuclease T1, an enzyme that cleaves 3'-5'-linked GpG bonds, but does not affect 2'-5'-linked bonds. The almost complete disappearance of product bands from the gel (data not shown) indicates that the oligomers formed on dC₁₀ and PNA-C₁₀ templates are predominantly 3'-5'-linked.

The PNA monomers are acyclic amino carboxylic acids that cyclize readily to lactams. Consequently, they are not suitable substrates for template-directed synthesis. Instead we used the PNA dimer G₂ as substrate (Fig. 1*c, III*). We chose 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) in the presence of excess-2-methyl imidazole as activating agent. This combination would yield 2-methyl imidazolides of the PNA dimers, carboxylic acid derivatives closely analogous to the activated nucleotides used in the previous experiments. The results of an experiment using dC₁₀ as template and PNA-G₂ as substrate are presented in Fig. 4*b*. A series of four product peaks corresponding to PNAs G₄, G₆, G₈ and G₁₀ are observed. The retention times of the first three are identical to those of authentic PNAs G₄, G₆, G₈ as shown by co-injection. The combined yield of oligomeric product as estimated from their ultraviolet absorption amounts to 87% of the input of C₁₀ template. No substantial peaks corresponding to larger oligomers are detected. Comparison with the results of the non-template reactions (Fig. 4*a*) strongly suggests that G₂ dimers line up on the C₁₀ template,

FIG. 2 Oligomerization of guanosine 5'-phosphoro(2-methyl)imidazole (2-MelmpG) without template (a), with dC₁₀ as template (b), and with PNA-C₁₀ as template (c, d), analysed after 2 days (a–c) or 6 days (d). Reaction conditions: 0 °C; 0.1 M 2-MelmpG; 1.2 M NaCl; 0.2 M MgCl₂; 0.005 M template; and 0.2 M 2,6-lutidine buffer (pH 7.9 at room temperature). Reaction solutions were prepared in 0.7 ml Eppendorf tubes in 5 µl volumes. First stock solutions of NaCl and MgCl₂ with or without the template were coevaporated to dryness. To start the reaction, the residue was redissolved in a freshly prepared solution of 2-MelmpG in 2,6-lutidine buffer. At appropriate times, 1 µl reaction solution was added to 100 µl of an aqueous solution containing 0.02 M HCl and 0.005 M EDTA. The resulting solution (pH 2.8–3.0) was kept at 37 °C for 24 h to hydrolyse surviving phosphoro-imidazoles and then neutralized with aqueous NaOH. This solution (5 µl) was mixed with 1 ml starting buffer (pH 12) and analysed by high-performance liquid chromatography (HPLC) on RPC-5 as previously described¹⁵. The reaction products were eluted with a linear gradient of NaClO₄ (pH 12, 0–0.06 M, 90 min). The numbered peaks correspond to oligomers with all 3'-5'-linkages.



and are linked together in a template-directed reaction (Fig. 1c, III). Longer PNA sequences which could be formed if product oligomers bridged two template molecules are not observed in this system, as they sometimes are with nucleotide substrates¹¹.

When we attempted to oligomerize the PNA dimer G₂ on the PNA template C₁₀ under our standard conditions we obtained a totally unexpected result: the template inhibited the oligomerization. The effect if not large (Fig. 4c), but it is reproducible. This suggests that the PNA-C₁₀:PNA-G₂ double helix has a somewhat different structure from the corresponding RNA:PNA double helix, and that in the PNA structure the amino and activated carboxyl groups of adjacent residues are held in a configuration that is unfavourable for reaction.

Experience with the RNA:RNA and DNA:RNA systems has shown that the success of template-directed reactions with phosphorimidazole substrates depends on the precise structure of the imidazole moiety¹². It was only by searching through many imidazole derivatives that we discovered one, 2-MelmpG, that undergoes efficient, regiospecific oligomerization on a poly(C) template. This optimization is likely to hold only for duplexes that adopt the RNA structure; some other imidazole might work better with PNA. We decided, therefore, to repeat the oligomerization reaction of PNA-G₂ using imidazole in place of 2-methylimidazole. The results are presented in Fig. 4d, e.

Comparison of Fig. 4c, e, shows that, in the presence of imidazole (e) rather than 2-methylimidazole (c), PNA-C₁₀ does facilitate the synthesis of PNA-G₄ and longer G oligomers. The effect is not dramatic but is readily reproduced. Thus, as in our earlier experiments with RNA templates and substrates, the nature of the imidazole moiety of the activated derivative has a profound influence on the outcome of the reaction. We plan to search a 'library' of imidazoles to see if we can find one that makes possible efficient template-directed ligation reactions of PNAs.

The 'genetic takeover' of the replicating system by another was first discussed by Cairns-Smith. He has argued at length that the first genetic system was a replicating clay, and that it was taken over by a replicating organic polymer¹³. In this and other examples where the structures of the replicating systems are unrelated³, it is difficult to see how information could be transferred from one system to the other. Our experiments are not relevant to this kind of genetic takeover.

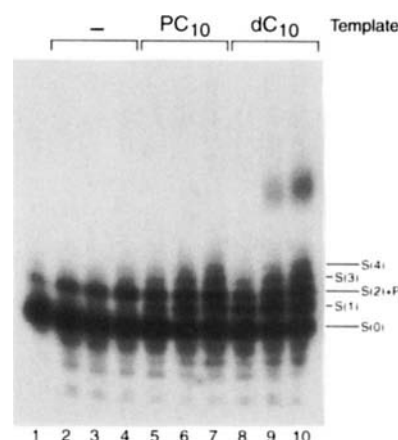
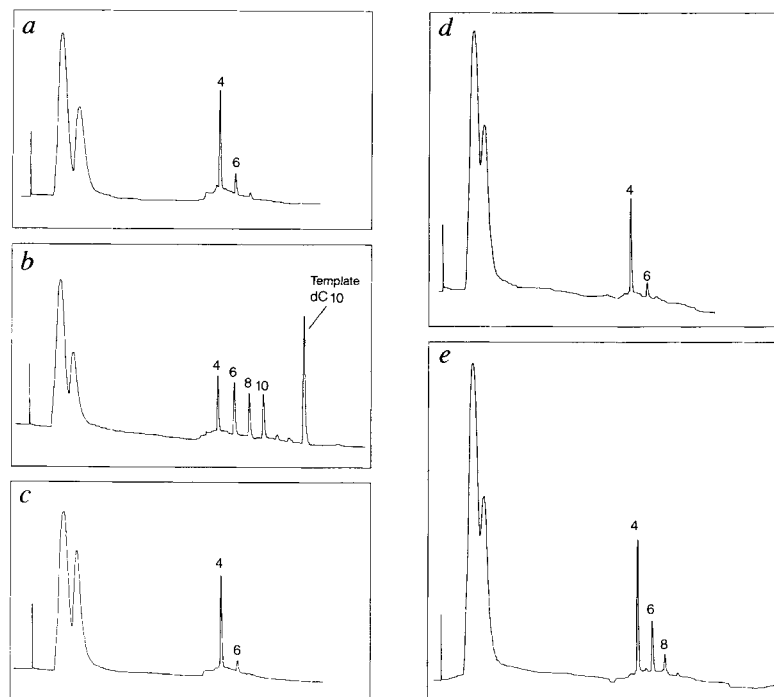


FIG. 3 Primer extension reaction of [³²P]-dG₄rG with 2-MelmpG without template (lanes 2–4) with PNA-C₁₀ template (lanes 5–7) or with dC₁₀ template (lanes 8–10) analysed after 4 h (lanes 2, 5, 8), 1 day (lanes 3, 6, 9) and 2 days (lanes 4, 7, 10) by polyacrylamide gel electrophoresis (PAGE) followed by autoradiography (lane 1 corresponds to the primer). Reaction conditions: 4 °C; 20 nM primer; 2 µM template; 1.2 M NaCl; 0.2 M MgCl₂; 0.05 M 2-MelmpG in 0.2 M 2-methylimidazole buffer (pH 8 at 4 °C). S(0) designates the starting material ([³²P]-dG₄rG), S(1) the first extension product, S(2) the second extension product, and so on. P designates the pyrophosphate-capped primer, which coelectrophoreses with S(2). Template-directed reactions were prepared in 0.7 ml Eppendorf tubes in 20 µl volumes by coevaporating stock solutions of NaCl, MgCl₂ and the primer (about 700,000 c.p.m., 0.2 pmole) to dryness. The residue was redissolved in 10 µl 0.4 M 2-methylimidazole buffer (pH 8 at 4 °C) and 7 µl water. The solution was heated to 95 °C for 10 min and then slowly cooled down, first to room temperature (1 h) and then to 4 °C (30 min). The reaction was initiated by adding 3 µl freshly prepared solution of 0.33 M 2-MelmpG. The tubes were then kept at 4 °C. Sampling: at given times, 2 µl reaction solution were added to 100 µl aqueous solution containing 0.01 M EDTA and 0.02 M HCl. The resulting solution (pH 4) was kept at 37 °C for 2 h to hydrolyse excess 2-MelmpG and then neutralized with 2 µl 1M NaOH. Samples (20 µl) of this solution were evaporated to dryness, redissolved in 5 µl water and 15 µl loading buffer. Before loading on the gel the solution was heated to 95 °C and cooled quickly to 0 °C.

FIG. 4 Oligomerization of PNA-G₂ without template (a, d), with dC₁₀ template (b) or PNA-C₁₀ template (c, e) in the presence of 2-methyl imidazole buffer (a–c) or imidazole buffer (d, e), analysed after 6 h. Reaction conditions: 25 °C; 5 mM PNA-G₂; 0.5 mM template; 0.2 M EDC; 0.4 M buffer (2-methylimidazole or imidazole; pH 7 at 25 °C). Reaction solutions were prepared as described in Fig. 3 by coevaporating a stock solution of PNA-G₂ with or without the template. The reaction was initiated by redissolving the residue in 5 µl freshly prepared solution of 0.2 M EDC in buffer. HPLC analysis: 1 µl reaction solution was added to 1 ml starting buffer (pH 12) and heated to 95 °C for 15 min. The solution (60 µl) was then analysed by HPLC on RPC-5 (see Fig. 2). The reaction products were eluted with starting buffer for 5 min, followed by a linear gradient of NaClO₄ (pH 12; 0–0.08 M in 35 min). The numbered peaks correspond to PNA-G oligomers of the designated length.



A fundamentally different mechanism for genetic takeover has also been suggested². It is proposed that a preformed molecule of the ancestral genetic system acted as a template for the incorporation of the monomers of the second system, without loss of sequence information. Our experiments are a first step towards the demonstration that takeovers of this kind can occur. A PNA oligomer C₁₀ acts as a template for the regioselective oligomerization of 2-MelmpG while a DNA sequence dC₁₀ acts as template for the oligomerization of the PNA oligomer G₂. Many further experiments will be needed to determine the range of sequences that act as efficient templates. It is also important to find out whether heteropolymeric chains containing RNA and PNA monomers can be generated and, if they can, whether they act as templates.

PNA is an achiral molecule so it must catalyse the oligomerization of activated D- and L-nucleotides with equal efficiency. When short PNA oligomers are complexed with racemic nucleotides, it is likely that double helices will form which contain only D- or only L-nucleotides. Template-directed synthesis might then yield a mixture of homochiral L-oligomers and homochiral D-oligomers from a racemic substrate. When longer PNA templates are used, analogous reactions should lead to block-copolymers of all D- and all L-RNA sequences. However, enantiomeric cross-inhibition¹⁴ may well limit the efficiency of these processes. The use of PNA as a 'chemical mirror' is also a theoretical possibility. A D-oligonucleotide of defined sequence could be used as a template for the synthesis of PNA. The PNA in turn could be used to synthesize an L-oligonucleotide with the same sequence as the original D-oligonucleotide. □

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Polymer microstructures formed by moulding in capillaries

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THE formation of patterned structures on micrometre-length scales is essential for the fabrication of many electronic, optical and mechanical devices¹. Patterning technologies are well established for semiconductors and metals, but are relatively undeveloped for organic polymers (with the notable exception of the specialized polymers used in photolithography¹). Polymeric replicas of some structures have been formed by filling them with monomers which are subsequently polymerized^{2–5}. But these procedures have important limitations, in that they usually involve the destruction of the template structure, or the resulting structures are not sufficiently regular for most applications. Here we describe a general moulding procedure which does not suffer from these limitations. For the mould we use the continuous network of channels formed when a substrate and a patterned elastomeric master are placed in intimate contact. A low-viscosity polymer precursor is placed in contact with the network, which then fills spontaneously by capillary action. After cross-linking the precursor, the master is removed (and can be reused), leaving a patterned polymer layer; depending on the choice of substrate, patterned free-standing films can be similarly produced.

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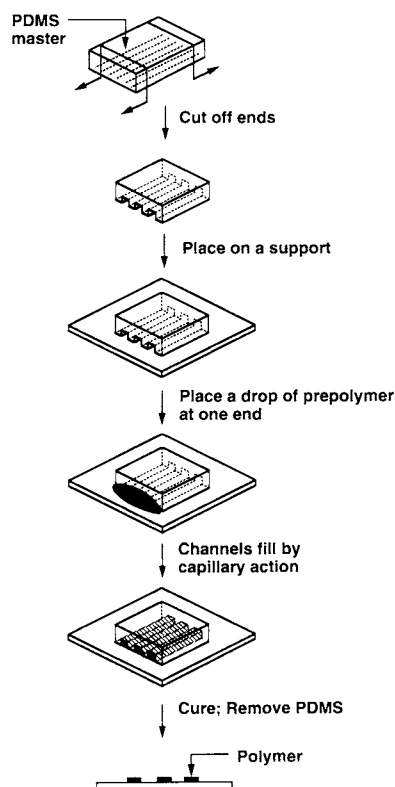


FIG. 1 Schematic diagram of capillary micromoulding of a pattern of parallel, rectangular channels. It is not necessary for both ends of the channels to be open: even if one end is closed, the channels fill with the prepolymer. The trapped air seems to escape by diffusing through the PDMS master.

Photolithography has successfully provided routes to planar and quasi-planar supported structures in the specialized polymers used as photoresists¹. Techniques for forming microstructures in structural and functional polymers would allow microfabrication of a broader range of organic materials. Here we describe a new procedure—micromoulding in capillaries (MIMIC)—for fabricating microstructures of polymeric materials (Fig. 1). We fabricate an elastomeric master that has a planar surface with a network of recessed channels by casting poly(dimethylsiloxane) (PDMS, Sylgard 184, Dow Corning) against a complementary relief structure (in turn prepared by photolithographic or non-photolithographic procedures)^{5–8}. We cut the ends of this master so that the network of channels is open and allows the liquid prepolymer to enter. The master is placed on a support; the patterns in the surface of the master form a network of channels. When a drop of a liquid prepolymer is placed at one open end of this network, the liquid spontaneously fills the channels by capillary action. The initial rate at which a prepolymer of polyurethane (J-91, Summers) moved along a channel with approximate cross-sectional dimensions $3.0 \times 1.5 \mu\text{m}$ over a solid support of Si/SiO₂ was $\sim 0.5 \text{ mm min}^{-1}$. The prepolymers were cross-linked thermally (at 65°C for 1–2 h, for thermally cured epoxies) or photochemically (thin PDMS films are optically transparent)⁹. Once the polymer had cross-linked sufficiently, the PDMS stamp could be removed from the support. These cross-linked polymers did not adhere to PDMS, and the patterned polymeric layer remained on the support.

This process generates negative replicas of the patterns on the surface of the master. Features having flat edges and acute angles can be prepared (Fig. 2a, b); the edge resolution obtained in the polymeric structure is determined by the PDMS stamp. We have

prepared samples with the complexity of those in Fig. 2 with macroscopic dimensions of several square centimetres.

MIMIC can also be used to fabricate free-standing films, using two procedures. In the first, the polymer structure is formed on a solid support and then freed by dissolving, melting or vaporizing that support. For example, Fig. 2c shows a complex patterned structure that has been removed from a Si/SiO₂ surface by dissolving the oxide layer in an aqueous solution of NH₄F/HF; the folding in the sample demonstrates its structural integrity. We have prepared other free-standing films by removing the support using appropriate conditions, such as dissolving

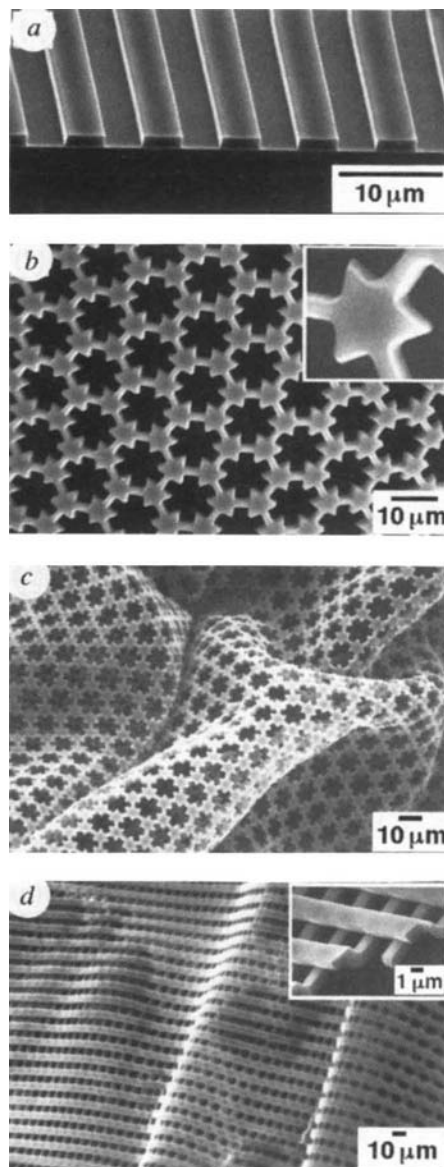


FIG. 2 Patterns fabricated by MIMIC. Patterns were sputtered with gold before imaging by scanning electron microscopy. The polymer used was photocured poly(methylmethacrylate). *a*, An oblique image of a fractured sample showing rectangular slabs of polymer on a gold film supported on Si/SiO₂. *b*, Image (captured at 30°) of more complex patterns on a silicon wafer. *c*, A free-standing film: the same film as in *b* was released from the surface by dissolving the SiO₂ layer in NH₄F/HF. The folding in this region of the sample was accidental, but illustrates the flexibility of the film. *d*, A free-standing polymeric structure fabricated in channels formed by conformal contact of two PDMS masters like those in *a*, using an orientation in which the grooves of the two masters were perpendicular. The two layers of the channels formed one interconnected polymeric structure (inset).

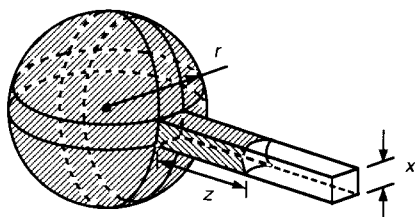


FIG. 3 A model for capillary micromoulding.

glass in HF, single-crystal NaCl and KCl in water, polymer films in solvents (for example, acetone for a film of a photoresist), or melting (paraffin film). In the second, the polymeric structure is formed between two PDMS masters. After filling with and cross-linking the precursor, the polymeric structure was removed from the master using Scotch tape or tweezers (Fig. 2d). This type of structure—two layers with independent structure in each—cannot be fabricated by photolithography in a single step.

The three components of the system used for MIMIC—the elastomeric master, the support, and the liquid prepolymer—must have certain characteristics. The master must be elastomeric, have a low surface free energy, and be unreactive towards the prepolymer. PDMS is sufficiently elastomeric that it makes conformal contact with supports that are rough on the 100-nm scale⁷; this contact limits the lateral spreading of the prepolymer by capillarity into the regions in contact with the stamp. Because the PDMS master and the support adhere without applying external force, the process reproduces features in the surface of the master accurately. Even the low interfacial free energy of the surface of the PDMS master ($\gamma_{\text{PDMS/air}} = 21.6 \text{ dyn cm}^{-1}$; ref. 10) is sufficient to maintain adhesion between the master and the support. The PDMS component has very low reactivity towards the prepolymers under the curing conditions used here. Its elasticity is sufficient to allow its separation from the polymeric microstructures; if it was not an elastomer, this separation could be difficult or impossible.

We have used many different supports in MIMIC, including Si/SiO₂ (both native and thermally grown), Ni/NiO, Ti/TiO₂, platinum, glass, gold, NaCl, gold or SiO₂ covered by self-assembled monolayers (SAMs), and films of organic polymers. It is often helpful that the surface energy of the support is higher than that of PDMS as this ensures selective adhesion of the polymer being moulded to the support (rather than to the master), but it may not be necessary. The ability to tune precisely the surface properties of SAMs on gold and SiO₂ (refs 11, 12) makes these supports particularly useful. The support need not be flat: we have successfully used curved surfaces (glass) with radii of curvature from 0.25 to 1.0 cm. A PDMS master formed as a thin (~100 μm) film can adhere to a curved surface without losing the fidelity of the stamp. The support can also be a polymeric surface (with or without recessed patterns) (Fig. 2d).

The liquid prepolymer should have low viscosity (<400 cP) to allow flow through the capillary; it should have small volume changes (<3%) on curing; and it should wet the support (or the master, or both) at least partially; complete wetting, however, is not necessary. We have used several prepolymers successfully, for example ultraviolet-curable poly(methylmethacrylate) (SK-9, Summers Optical), ultraviolet-curable epoxies (UV15, UV15-7, Master Bond), heat-curable epoxies (F113, F114, TRA-CON), ultraviolet-curable polyurethanes (J-91, Summers Optical; NOA 60, 71, 72, 73, 88, Norland), heat-curable polyurethane (NOA 133, Norland) and ultraviolet-curable poly(methylacrylate) (SJ-7, Summers Optical). Photocurable polymers were irradiated with an ultraviolet light (Canrad-Hanovia 450 W medium-pressure, mercury vapour lamp Type 7825-34) for 10–20 min at a distance of 1–2 cm from the sample. It is important that the prepolymer has low viscosity and that it cross-links under achievable conditions.

The most important concept of MIMIC is that the liquid organizes and forms patterns as a result of capillary forces. The changes in interfacial free energies for a liquid moving from a spherical drop (radius r) into a hollow tube with a square cross-section (width x) are given by equations (1) and (2) (Fig. 3)^{13, 15}. Here three surfaces are assumed identical (the PDMS) and one different (the support). The terms γ_{SV} , γ_{SL} and γ_{LV} are solid–vapour, solid–liquid and liquid–vapour interfacial free energies, respectively; θ and θ' are the contact angles of the liquid prepolymer on the surface of PDMS and the support.

$$\Delta G = \gamma_{\text{LV}} \Delta A^{\text{sphere}} - f(\gamma_{\text{SL}}, \gamma_{\text{SL}}) \Delta A^{\text{channel}} \quad (1)$$

$$= x^2 \Delta z \gamma_{\text{LV}} / r - [3x \Delta z (\gamma_{\text{SV}} - \gamma_{\text{SL}}) + x \Delta z (\gamma_{\text{SV}} - \gamma_{\text{SL}})] \quad (2)$$

$$\approx -[3x \Delta z (\gamma_{\text{SV}} - \gamma_{\text{SL}}) + x \Delta z (\gamma_{\text{SV}} - \gamma_{\text{SL}})] \quad (3)$$

$$\approx -x \Delta z \gamma_{\text{LV}} (3 \cos \theta + \cos \theta') \quad (4)$$

When $r \gg x$ and the term due to the drop of liquid prepolymer can be neglected, equation (2) becomes equation (3) (or the equivalent, equation (4)). These equations demonstrate that this process is dominated by the free energies of the solid–vapour and solid–liquid interfaces. Only when $\gamma_{\text{SV}} - \gamma_{\text{SL}}$ (and/or $\gamma_{\text{SV}} - \gamma_{\text{SL}}$) are negative (a situation that normally correlates with high γ_{LV}) will the capillary not fill. As an approximation, this capillary force is positive, that is, the liquid spontaneously fills the capillary, for any value of θ and θ' between 0 and 90°; the liquid wicks through the capillary when the liquid wets or partially wets the inside of the capillary¹⁶. The capillary force is negative for liquids with high values of γ_{SL} and γ_{SL} ($\theta > 90^\circ$ and $\theta' > 90^\circ$); these liquids do not fill the capillary. If $\theta > 90^\circ$ and $\theta' < 90^\circ$, the balance of forces becomes ambiguous. An example of this last case is water when the master is PDMS, and the support is a partially hydrophilic SAM ($-\text{COOCH}_3$ terminated) on gold ($\theta = 105^\circ$; $\theta' = 60^\circ$); we need to add surfactant or an organic solvent such as ethanol (~5% is sufficient) to the water to achieve flow through the capillary. The surface of PDMS may also be modified to increase the interfacial free energy and to ensure wetting by a liquid¹⁷. We usually allow the capillarity to fill the channels while they are oriented perpendicular to the force of gravity. If the channels were filled against (or with) the force of gravity, the force acting to fill the capillary will be reduced (or augmented) by $F' = x^2 h \rho g$, where h is the height of the liquid in the capillary, ρ the density of the liquid, and g the gravitational constant.

The surface tension and viscosity of the liquid, the size of the capillary, and the length of the channel determine the rate of liquid flow in the capillary (equation (5))¹⁵:

$$\frac{dz}{dt} = \frac{R \gamma_{\text{LV}} \cos \theta}{4 \eta z} = \frac{R (\gamma_{\text{SV}} - \gamma_{\text{SL}})}{4 \eta z} \quad (5)$$

where R is the hydraulic radius (the ratio between the volume of the liquid in the capillary section and the area of the solid and liquid interface), η the viscosity of the liquid, and z the length of the column of liquid. Our experimental results are qualitatively consistent with this relationship. Equation (5) indicates that the filling of very small capillaries will be slow because R will decrease as the dimensions of the capillary decrease. We have observed the filling of sections of capillary that were approximately $1.0 \times 0.1 \mu\text{m}$ in cross-section, but for a short distance only.

Capillary micromoulding has several useful differences from conventional photolithography as a method of forming polymeric microstructures. Photolithography requires two steps: forming (usually by spin coating) and patterning photoresist films. MIMIC requires one: forming and patterning the polymeric films occur simultaneously. Photolithography requires one exposure per structure; the master in MIMIC can be used many times, and photolithography is used only once (if at all) to make the master. Photolithography is limited to special classes of polymers and photosensitizers; MIMIC is applicable to most polymers with low viscosity. Photolithography is a two-dimensional

process: patterning layers with different thicknesses is impossible in a single step.

Micromoulding driven by capillarity is remarkable for its simplicity, for the fidelity with which the patterns present in the mould are preserved in the polymer, and for the effectiveness with which capillarity fills the channels. □

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Mutations in the palmitoyl protein thioesterase gene causing infantile neuronal ceroid lipofuscinosis

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NEURONAL ceroid lipofuscinoses (NCL) represent a group of common progressive encephalopathies of children which have a global incidence of 1 in 12,500 (ref. 1). These severe brain diseases are divided into three autosomal recessive subtypes, assigned to different chromosomal loci^{2–4}. The infantile subtype of NCL (INCL), linked to chromosome 1p32, is characterized by early visual loss and rapidly progressing mental deterioration, resulting in a flat electroencephalogram by 3 years of age; death occurs at 8 to 11 years⁵, and characteristic storage bodies are found in brain and other tissues at autopsy⁶. The molecular pathogenesis underlying the selective loss of neurons of neocortical origin has remained unknown. Here we report the identification, by positional candidate methods, of defects in the palmitoyl-protein thioesterase gene in all 42 Finnish INCL patients and several non-Finnish patients. The most common mutation results in intracellular accumulation of the polypeptide and undetectable enzyme activity in the brain of patients.

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The defective gene underlying INCL was isolated using a positional cloning strategy⁷. We initially assigned the disease locus (CLN1) to human chromosome 1p32 (ref. 3) using a random linkage approach, and further restricted it to a region of 4 cM between the *L-myc* gene and the marker MS154 (ref. 8). Linkage disequilibrium mapping suggested that the CLN1 locus would lie in the vicinity of a microsatellite marker, HY-TM1 (ref. 9), located 16 kb centromeric to *L-myc*, and haplotype analysis suggested only one founder mutation in the Finnish population⁸. We established long-range restriction maps¹⁰ and constructed a contiguity consisting of several genomic clones spanning the critical chromosomal region for CLN1 at 1p32. Figure 1a shows this physical map, including the locations of the known *L-myc* (ref. 11), *rlf* (ref. 12) and *COL9A2* (ref. 13) genes. We also identified several novel CpG islands, indicating the presence of at least four new genes within the 600-kb region proximal to *L-myc*.

Simultaneous with positional cloning efforts, a palmitoyl-protein thioesterase (PPT) gene was mapped to human chromosome 1p32 by fluorescent *in situ* hybridization (FISH) to metaphase chromosomes (data not shown). The PPT gene encodes an enzyme thought to be involved in the catabolism of lipid-modified proteins¹⁴, and was considered to be a potential candidate for the defective gene.

Several long-range clones in the critical CLN1 region contained the PPT gene, by hybridization to a PPT complementary DNA probe. To locate the PPT gene precisely on the physical map, we hybridized a PPT genomic clone (Fig. 1b, shown in red) and regionally well-assigned P1 and λ clones (red and green) to extended DNA fibres. The PPT gene was positioned 70 kb telomeric to the 5' end of *rlf* (rearranged *L-myc* fusion). This finding, combined with the results of pulsed-field gel electrophoresis (data not shown), assigned the PPT gene under one of the CpG islands in the critical CLN1 region (Fig. 1a).

Direct sequencing of cDNAs derived from brain RNA of two Finnish INCL patients and one control individual demonstrated a mis-sense transversion of A to T at nucleotide position 364, resulting in the substitution of tryptophan for arginine at position 122 in the protein (Fig. 1c). The rest of the coding region was identical between control and patients. Arg 122 is immediately adjacent to a lipase consensus sequence¹⁵ that contains the putative active-site serine of PPT¹⁶. The frequency of this nucleotide substitution was determined in 42 Finnish and 17 INCL families from Europe and the United States, and in 200 Finnish control individuals (Table 1). In 40 of the 42 Finnish families, patients were homozygous and parents heterozygous for the substitution, an observation in accordance with the prediction of one major INCL mutation in the Finnish population resulting from a founder effect, and subsequent isolation. In two

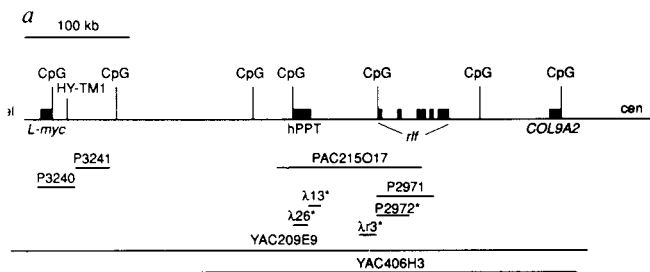
TABLE 1 Genotypes at nucleotide position 364 in human PPT

Sample	AA*	AT*	TT*
Finnish INCL patients (n = 42)	0	2	40
Finnish obligatory carriers (n = 84)	2	82	0
Finnish controls (n = 200)	197	3†	0
Non-Finnish INCL patients (n = 17)	13	2	2
Non-Finnish obligatory carriers (n = 34)	28	6	0

The nucleotides at the mutation site in the PPT gene were identified by the solid-phase minisequencing method¹⁸ using human-specific PPT primers. The PCR primers were 5'-TCTCCAGGGAGGCCAATTC-3' (biotinylated) and 5'-GTGAAGGCATCTCTGAGCCA-3', and the minisequencing primer was 5'-CGTAGAGCTCGTGACGGG-3'.

* AA, homozygous normal; AT, heterozygous; TT, homozygous mutant.

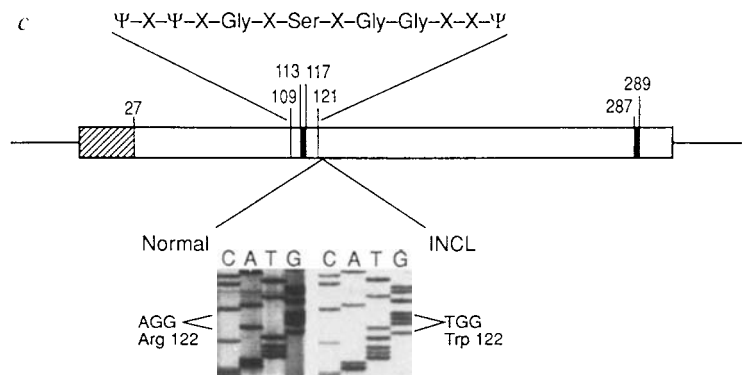
† INCL carriers identified among the control individuals.



λ26 + λ13

λr3 P2972

FIG. 1 a, Physical map of the CLN1 region at 1p32. Known genes in the region are indicated by boxes, and genomic clones by horizontal bars. CpG islands and the marker HY-TM1 are shown as vertical bars. Clones used for *in situ* hybridization are marked with asterisks. **b**, Assignment of PPT to a region 70 kb telomeric to *rlf* by fluorescent *in situ* hybridization to extended DNA (fiberFISH)¹⁹ using the PPT probes λ13 and λ26 (red) and the *rlf* clones λr3 (green) and P2972 (red). The estimation of distance separating *rlf* and PPT is based on the size of genomic clones used as hybridization probes. **c**, The INCL mutation. A linear representation of the PPT polypeptide is shown above a sequencing autoradiogram showing the single A to T transversion that causes substitution of tryptophan for arginine at amino acid residue 122. The change is adjacent to the consensus lipase site, shown at the top of the diagram. Ψ indicates any hydrophobic amino acid. Conserved thioesterase sequences (113–117 and 287–289) and the first amino acid in the processed mature protein (27) are indicated. **METHODS**. The yeast artificial chromosome (YAC) clones 209E9 and 406H3 have been described previously¹⁰. The P1 clones P3240 and P3241 were obtained from Genome Systems Inc. (St. Louis, MO) by PCR screening with the primer pair generated from the L-myc-positive cosmid clone ICRFc112L0386, and the clones P2971 and P2972 were obtained in a similar fashion with the primer pair generated from the *rlf* 5' genomic clone. The P1 derived artificial chromosome²⁰ clone 215017 was isolated using the *rlf* primer pair. Human PPT clones were isolated by hybridization screening from a lymphoblastoid genomic DNA library in bacteriophage λ (Stratagene) using the human PPT cDNA as a probe. FiberFISH analysis was performed as previously described¹⁹.



To obtain the human cDNA encoding PPT, a human brain cDNA library in bacteriophage λ was screened under conditions of reduced stringency with a probe derived from the bovine PPT cDNA¹⁴. The nucleotide sequence of human PPT cDNA has been deposited in the GenBank database (accession no. L42809). For mutation analysis, total RNA from control or INCL brain was reverse-transcribed and PCR amplified using human-specific PPT primers (5'-GTGGTGACAC-AGCGAAGATG-3' and 5'-AACTGTCTCCCATGTGGTTTG-3') under standard conditions. The nucleotide sequence was determined by solid-phase sequencing²¹.

Finnish INCL families, the patients were heterozygous for the mutation of A to T at position 364 at the genomic level, but when cDNA transcribed from total RNA of patient lymphoblasts was analysed, only the mutated allele was identified, demonstrating a null allele on the other chromosome. The existence of the mutation leading to a null allele was confirmed by polymerase chain reaction amplification from genomic DNA and cDNA after reverse transcription using the same primer set (described in Table 1). Of 200 control Finnish individuals, 3 were heterozygous for the A to T mutation, which is consistent with the estimated carrier frequency in Finland (1 in 70). Of 17 non-Finnish INCL patients examined, two (one German and one Estonian) were homozygous, and two (one German and one Swede) were heterozygous for the A to T mutation, the remaining patients being homozygous for the wild-type allele at position 364. A third mutation, an A to T transversion, was found at nucleotide position 163 in one PPT allele of a British INCL patient leading to an early stop codon at position 55.

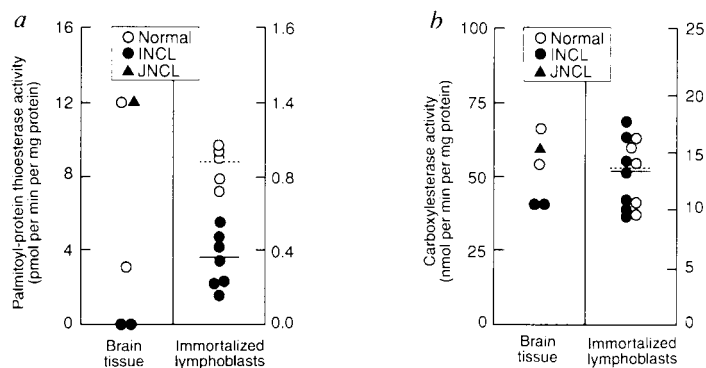
PPT activity was undetectable in brain tissue from two Finnish INCL patients (Fig. 2a), but normal in the brain of one patient with the juvenile-onset form of NCL, another subtype of NCL disorders assigned to chromosome 16p (ref. 2). Immortalized lymphoblasts derived from the peripheral blood cells of INCL patients and controls were also assayed for PPT activity. Although the activity in normal lymphoblasts was found to be rather low (<10%) compared with activity in normal brain, there was a marked difference in lymphoblast PPT activity between patients and controls, whereas an assay for nonspecific carboxyl-esterase activity in the brains and lymphoblasts of controls and INCL patients found no significant differences (Fig. 2b).

The primary cellular consequences of the main INCL mutation were studied by expression of the mutant and wild-type cDNAs in COS-1 cells. PPT is normally found both associated with cells and secreted into the tissue culture medium¹⁴. However, in a 3-h pulse experiment the mutant polypeptide was found in the cells but was not secreted into the medium (Fig. 3a, bottom). Furthermore, the cell-associated mutant polypeptide was inactive (Fig. 3a, top). Immunofluorescent staining of COS-1 cells expressing either wild-type or mutant PPT revealed striking differences (Fig. 3b): the wild type showed strong juxtanuclear staining and a punctate pattern throughout the cytoplasm, consistent with staining of the Golgi apparatus and secretory vesicles, whereas the mutant PPT showed a reticular pattern typical of endoplasmic reticulum (ER) staining. This suggests that most of the mutant polypeptide is trapped in the ER.

That three independent mutations were found in the PPT gene of INCL patients strongly suggests that defects in this gene cause INCL. Further, the expected high frequency of the A to T mutation at position 364 in Finnish disease alleles and the absence of enzyme activity in INCL brain tissue support this suggestion. Finally, deficient enzyme activity and abnormal intracellular localization of the expressed recombinant mutant PPT polypeptides provide further evidence that the mutation in the PPT gene ultimately results in the manifestations of the disease.

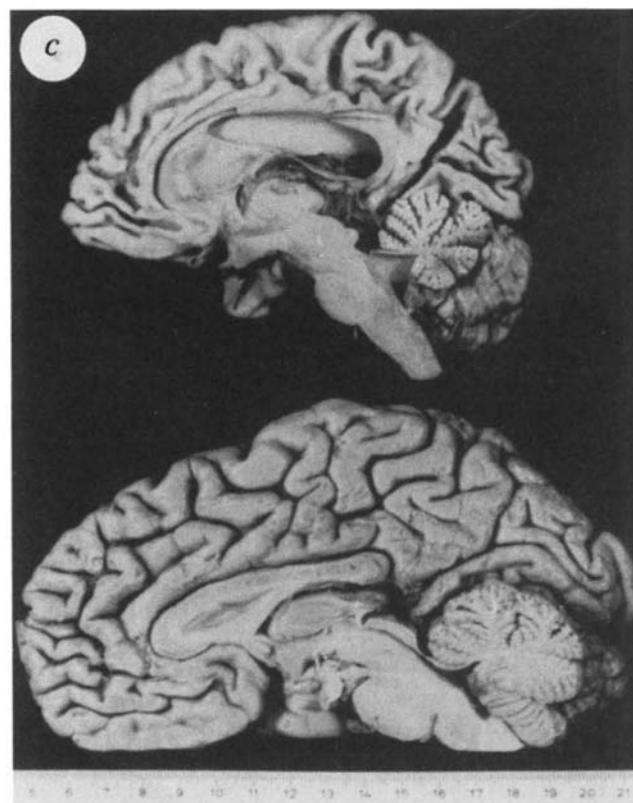
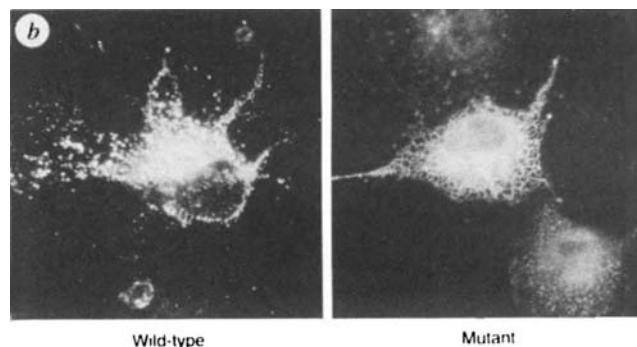
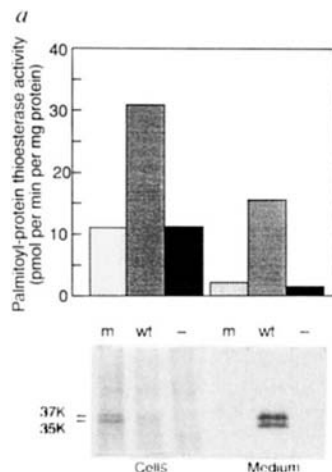
How the defect in the PPT enzyme leads to the degeneration of cortical neurons is currently unknown. It is remarkable that the manifestations of the disease are almost entirely limited to the neocortex (Fig. 3c), rather than the brain stem, spinal cord or other tissues. PPT must be important for the maintenance of

FIG. 2 Absent or decreased PPT activity in brain tissue and peripheral blood lymphocyte-derived cells in INCL patients. **a**, PPT activity in soluble extracts of human brain or whole-cell extracts of Epstein–Barr virus-transformed lymphoblasts from normal individuals (open circles), INCL patients (filled circles) and a juvenile NCL (JNCL) patient (filled triangle). PPT activity was undetectable (<0.05 pmol per min per mg protein) in INCL brain tissue. Lymphoblast PPT activity was 0.89 ± 0.05 (s.e.) pmol per min per mg protein in normals and 0.37 ± 0.05 (s.e.) pmol per min per mg protein in INCL patients ($P < 0.003$, paired Student's *t*-test). **b**, Carboxylesterase activity in normal and NCL brain and lymphoblastoid cells. Carboxylesterases are nonspecific serine hydrolases that share limited sequence homology with PPT. The activities in normal, INCL and JNCL patients were not significantly different (13.3 ± 2.5 versus 13.2 ± 3.0 nmol per min per mg protein). **METHODS.** Human brain samples collected at autopsy were homogenized in buffer, and a soluble fraction for enzyme assays was obtained by differential centrifugation as described²². Whole-cell extracts of lymphoblastoid cells were prepared as described¹⁴. PPT assays were performed using [³H]palmitate-labelled H-Ras as the substrate, with preincubation of samples in 4 mM PMSF to inhibit PMSF-sensitive PPT activity²³. The PMSF-sensitive activity was a small proportion (7%) of the total PPT activity in normal brain samples, but a



significant portion of the total activity in lymphoblastoid cells. The PMSF-sensitive activity in lymphoblastoid cells did not differ significantly between normal and INCL samples (0.47 ± 0.03 versus 0.43 ± 0.03 pmol per min per mg protein). Carboxylesterase assays were done as previously described²³ using *p*-nitrophenylacetate as a substrate.

FIG. 3 Effect of the INCL mutation on recombinant hPPT enzyme activity, secretion and subcellular localization. **a**, PPT enzyme activity and immunoprecipitated PPT in COS-1 cells transfected with Arg122Trp mutant (m), wild-type (wt) and control vector (–). The transfected cells were either collected for enzyme assays (top) or pulse-labelled with [³⁵S]-methionine (see Methods) (bottom). PPT appears as a doublet of relative molecular mass (*M_r*) 37K and 35K owing to differential glycosylation¹⁴. The mutant enzyme is inactive and is not secreted into the culture medium. **b**, Immunofluorescence analysis of wild-type and mutated PPT polypeptide in COS-1 cells. The cells expressing mutant PPT show a reticular staining pattern, whereas the cells expressing normal PPT show a strong juxtanuclear staining as well as a punctate pattern throughout the cytoplasm. **c**, Sagittal section of a brain from an 8-year-old INCL patient at autopsy (top) and an age-matched control (bottom). Scale is in centimetres.



METHODS. The wild-type PPT cDNA plasmid was obtained by reverse transcription and PCR amplification of the coding region from total brain RNA followed by ligation into an SVpoly expression vector²⁴. The mutant plasmid was constructed by oligonucleotide-directed site-specific mutagenesis²⁵ in which A was changed to T at position 364. Both constructs were verified by dideoxynucleotide sequencing of the entire coding region²⁶. After COS-1 cell transfection²⁷, cells and medium were either collected at 48 h post-transfection and processed for PPT enzyme assays¹⁴ or pulse-labelled with 200 μ Ci ml^{-1} of [³⁵S]-methionine for 3 h. The medium was collected, and the cells collected by trypsinization, washed twice with PBS (phosphate buffered saline) and lysed by freeze-thawing in PBS with 1% Triton X-100. Radiolabelled PPT polypeptides were immunoprecipitated using a rabbit anti-bovine PPT antibody¹⁴ and immuno-precipitin reagent (GIBCO BRL)²⁸. The precipitated proteins were separated by 14% sodium dodecyl sulphate-polyacrylamide gel electrophoresis²⁹ (SDS-PAGE) and visualized by fluorography using Amplify enhancer (Amersham). Exposure times were 2 (cells) and 5 (medium) days. Immunofluorescence staining of transiently transfected and saponin-permeabilized COS-1 cells was performed as previously described³⁰ using rabbit anti-bovine PPT antibody¹⁴ ($5 \mu\text{g ml}^{-1}$) followed by staining with a 1:40 dilution of rhodamine-conjugated swine anti-rabbit IgG. The coverslips were viewed with a Zeiss Axiophot immunofluorescence microscope at $\times 630$ magnification.

cortical function. It removes thioester-linked fatty acyl groups from S-acylated proteins, but is also a palmitoyl-CoA hydrolase *in vitro*¹⁴. Identification of the *in vivo* substrate(s) for PPT will help our understanding of the pathophysiology of INCL.

We know of only one example of lipid modifications of proteins being involved in the pathogenesis of an inherited human disease, choroideraemia, which is caused by a defect in protein prenylation¹⁷. It is intriguing that both choroideraemia and INCL involve defective cysteine lipid modifications, and both have choroid-retinal degeneration as prominent manifestations of disease, although the significance of these observations is unclear. Characterization of causative mutations in other representatives of NCL disorders with somewhat less dramatic symptoms than INCL will show whether they also involve defects in lipid modification of proteins. □

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Brain regions associated with retrieval of structurally coherent visual information

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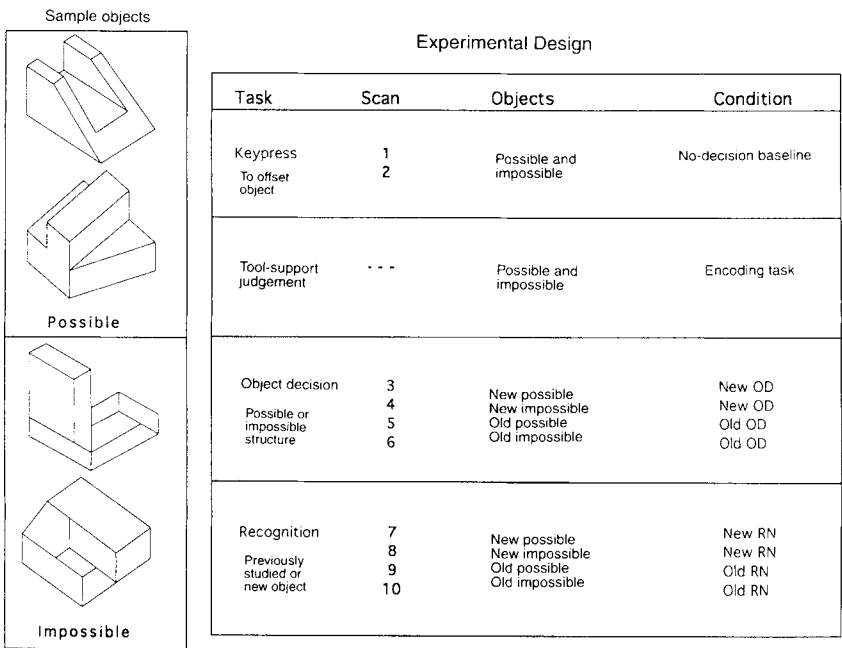
AN object's global, three-dimensional structure may be represented by a specialized brain system involving regions of inferior temporal cortex^{1–3}. This system's role in object representation can be understood by experiments in which people study drawings of novel objects with possible or impossible three-dimensional structures, and later make either possible/impossible object decisions or old/new recognition decisions about briefly flashed studied and non-studied objects. Although object decisions about possible objects are facilitated by prior study, there is no corresponding facilitation for impossible objects, thereby implicating a system that is specifically involved in the representation of structurally coherent visual objects⁴. Here we show, by positron emission tomography (PET), that increases in blood flow in inferior temporal regions are associated with object decisions about possible but not impossible objects, and that there are increases in the vicinity of the hippocampal formation associated with episodic recognition of possible objects.

Ten 31-slice PET images of regional cerebral blood flow in 16 healthy females were obtained using the ECAT 951/31 scanner

(Siemens, Knoxville, Tennessee), 45 mCi intravenous bolus injections of H₂¹⁵O, and 60-s scans as they viewed 50-ms presentations of individual possible or impossible objects. During the first two scans, subjects pressed a button with their index finger when an object disappeared from the screen, providing no-decision baseline images that controlled for visual stimulation and movement. Subjects then studied a series of 20 possible and 20 impossible objects in the absence of a scan. During the next four scans, subjects made decisions about whether the objects were possible or impossible during four separate blocks of 20 old possible, 20 new possible, 20 old impossible, and 20 new impossible objects. The final four scans used the same arrangement of blocks, but subjects made old/new recognition memory decisions (Fig. 1). Despite procedural modifications necessitated by the demands of PET, behavioural data concerning object decision and recognition performance are in close agreement with previous results (Table 1). For each subtraction described below, automated algorithms were used to align the 10 PET images from each subject, spatially transform them into the coordinates of a standard brain atlas, control for variations in whole brain measurements, compute normalized *t*-score maps of significant increases in regional blood flow (maximal *t*-score > 2.58, *P* < 0.005, uncorrected for multiple comparisons), and superimpose the maps onto the subjects' spatially transformed and averaged magnetic resonance images^{5,8}.

To examine blood flow increases associated with object decisions about old and new, structurally coherent and structurally incoherent objects, the no-decision baseline scan was subtracted from the new and old object decision scans for possible and impossible objects, respectively. Object decisions about possible objects were associated with significant blood flow increases in the inferior temporal gyrus and the adjacent fusiform (occipito-temporal) gyrus (Fig. 2), bilaterally for old objects and on the right for new objects. In contrast, object decisions about old and new impossible objects were not associated with significant blood flow increases in the inferior temporal or fusiform gyri; the only exception was a trend (*P* < 0.05) in the right inferior temporal region in the new-object decision condition. To examine blood flow increases associated with changes in object decisions about structurally coherent objects as a result of studying them during the tool/support encoding task (priming effects; see Fig. 1, Table 1), the new possible-object decision scan was subtracted from

FIG. 1 Examples of objects used in the experiment are shown on the left. Possible objects are structures that could exist in the three-dimensional world. Impossible objects contain local edge or surface violations and hence could not be constructed as connected, three-dimensional solids. The overall experimental design is displayed on the right. Ten separate scans were used, each involving computerized presentation of 20 consecutive objects according to the following sequence: (1) a cross-hair fixation point for 250 ms; (2) a blank interval of 100 ms; (3) an object at central fixation for 50 ms followed by a key-press response; and (4) an inter-trial delay of 2,500 ms. The no-decision baseline scans (1 and 2) served as a control for visual exposure to the objects and actuation of a key-press response. Subjects next engaged in an encoding or study task, during which no scanning occurred. They saw 40 objects (20 possible and 20 impossible), presented individually in a random sequence, for 5 s each. Subjects indicated whether each object would best be used as a tool or for support. Previous research has shown that the tool/support judgement is one of several encoding tasks that produce significant effects of study, known as priming effects, on the object decision task²⁹. Object decisions (OD) were assessed in the next four scans. During two of these scans (3 and 4; new possible and new impossible), subjects made decisions of possible/impossible about objects that had not appeared previously at any point in the experiment; during the other two scans (5 and 6; old possible and old impossible), they made the same decisions about objects that had appeared during the encoding task. Finally, episodic recognition (RN) was assessed by using a similar arrangement of four scans, except that subjects indicated whether or not they remembered seeing an object during the encoding task. The new possible and new impossible scans (7 and 8) each used objects



that had not appeared previously in any phase of the experiment, whereas the old possible and old impossible scans (9 and 10) used objects that had appeared during the encoding task and during scans 5 or 6 of the object decision task. Assignment of objects to conditions and order of scans within conditions was completely counterbalanced for 12 subjects and partly counterbalanced for 4 subjects.

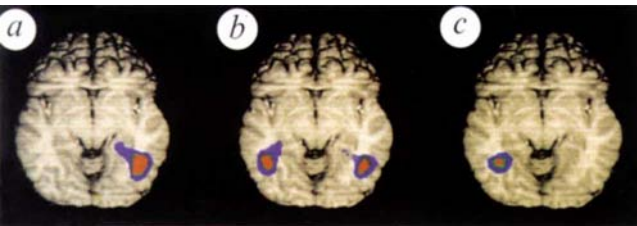


FIG. 2 For possible visual objects: a, new-object decision minus no-decision baseline; b, old-object decision minus no-decision baseline; and c, old-object decision minus new-object decision subtractions revealed significant blood flow increases in the vicinity of right, bilateral, and left inferior temporal and fusiform gyri, respectively. For each subtraction, automated algorithms were used to characterize significant increases in regional blood flow (those with a maximal normalized *t*-value > 2.58, *P* < 0.005, uncorrected for multiple comparisons⁶⁻⁸). For a-c, normalized *t*-value maps were superimposed onto a magnetic resonance image which was transformed into the coordinates of a brain atlas^{5,7}, volume rendered, and resected posterior to a coronal plane through the anterior commissure to reveal inferior temporal blood flow increases in a horizontal section 8 mm inferior to a plane through the anterior and posterior commissures. Increases in inferior temporal blood flow are shown in red, green and blue, which correspond to normalized *t*-values greater than 2.58, 2.33 and 1.64, and uncorrected probabilities less than 0.005, 0.01 and 0.05, respectively. Atlas coordinates and *t*-values of these and other significant blood flow increases are available from the author on request.

FIG. 3 For possible visual objects: a, new-recognition memory minus no-decision baseline; b, old-recognition memory minus no-decision baseline; and c, old-recognition memory minus new-recognition memory subtractions revealed significant blood flow increases in the vicinity of the left, bilateral and right hippocampus, respectively. For each subtraction, automated algorithms were used to characterize significant increases in regional blood flow (those with a maximal normalized *t*-value > 2.58, *P* < 0.005, uncorrected for multiple comparisons⁶⁻⁸). For a-c, normalized *t*-value maps were superimposed onto a magnetic resonance image which was transformed into the coordinates of a brain atlas^{5,7}, volume rendered, and resected posterior to a coronal plane through the anterior commissure to reveal hippocampal blood flow increases in a horizontal section 8 mm inferior to this plane in b and c. Increases in hippocampal blood flow (and additional increases in the vicinity of the parahippocampal gyrus and midbrain in b) are shown in red, green and blue, which correspond to normalized *t*-values greater



than 2.58, 2.33 and 1.64, and uncorrected probabilities less than 0.005, 0.01 and 0.05, respectively. Atlas coordinates and *t*-values of these and other significant blood flow increases are available from the author on request.

the old possible-object decision scan. This comparison revealed significant blood flow increases in the left inferior temporal/fusiform region (Fig. 2). When the same subtraction was performed for impossible objects, there were no significant blood flow increases in the inferior temporal and fusiform gyri. Although these findings suggest an association between priming of possible objects and left inferior temporal/fusiform blood flow increases, they could also be due to more possible but not more impossible objects being perceived as structurally coherent in the old-object decision condition than in the new-object decision condition.

Post hoc analyses were performed to characterize more directly those blood flow increases that were preferentially related to possible versus impossible objects, and those that were preferentially related to the left versus right hemisphere. Right inferior temporal and fusiform blood flow increases during object decision for possible objects were greater than those in the left hemisphere ($P < 0.005$), and were also greater than those in the right hemisphere during object decision for impossible objects ($P < 0.05$). Left inferior temporal and fusiform blood flow increases associated with studying possible objects were greater than those in the right hemisphere ($P < 0.005$), and were also greater than those in the left hemisphere during the same comparison for impossible objects ($P < 0.05$).

These findings are consistent with our proposal that a system involving inferior temporal regions is involved in the representation of a visual object's global, three-dimensional structure. Studies with non-human primates implicate inferior temporal regions in computing size-invariant representations of global object structure^{1,3,9}, and studies with human volunteers show that effects of prior study on object decisions about possible objects are size invariant¹⁰ and depend on encoding of global object structure⁴. Several kinds of evidence implicate the fusiform gyri in high-level object recognition^{11,12} and global or holistic visual representation^{13,15}.

For impossible objects only, both the old-object decision and new-object decision minus no-decision baseline comparisons revealed significant blood flow increases in the vicinity of the left hippocampal formation and the pulvinar bilaterally. The hippocampal formation has been linked previously with encoding of novel or unexpected stimuli¹⁶⁻¹⁹ and the pulvinar has been implicated in controlling attention to visually salient stimuli²⁰. Accordingly, these increases could reflect encoding of the novel or unusual features of impossible objects.

To examine blood flow increases associated with the recognition task, each no-decision baseline (possible or impossible) was subtracted from the corresponding old or new recognition scan, respectively (Fig. 3). For both possible and impossible objects, the new recognition minus no-decision baseline comparison revealed significant blood flow increases in the vicinity of the hippocampal formation bilaterally, predominantly on the left. The old-recognition minus baseline comparisons also revealed significant blood flow increases in the vicinity of the hippocampal formation, bilaterally for possible objects and predominantly on the left for impossible objects. For impossible objects only, these comparisons again revealed significant blood flow increases in the vicinity of the pulvinar bilaterally. Post hoc comparisons indicate that blood flow increases in the left hippocampal formation during the new-object decision, old-object decision, new recognition, and old recognition minus no-decision baseline comparisons for impossible objects were greater than those in the right ($P < 0.05$), and were also greater than those in the left during the same comparisons for possible objects ($P < 0.05$).

To examine blood flow changes specifically associated with memory for the appearance of an object in the study list, here referred to as episodic memory, the new-recognition condition was subtracted from the old-recognition condition (Fig. 3). For possible objects, this comparison revealed blood flow increases in the vicinity of the right hippocampal formation, left parahippocampal and fusiform gyri, the midbrain, and left dorsolat-

TABLE 1 Object decision and recognition performance

Task	Possible objects		Impossible objects	
	Old	New	Old	New
Object decision	0.67	0.56	0.63	0.58
Old/new recognition	0.82	0.33	0.68	0.36

For the object decision test, the table entries refer to the proportion of correct object decisions for old objects, which appeared previously during the encoding task, and the proportion of correct object decisions for new objects, which had not appeared during the encoding task. For possible objects, subjects made more correct decisions about studied than non-studied objects ($t(15) = 3.01$, $P < 0.005$), whereas, for impossible objects, significant differences between studied and non-studied objects were not observed ($t(15) < 1$). Nearly identical results were obtained in a purely behavioural pilot study using identical test procedures, carried out before the PET study. These findings replicate previously published results from a variety of conditions in numerous experiments^{2,4,10,29}. Overall levels of object decision accuracy for new and old objects are also closely comparable to data from previously published behavioural studies. For the yes/no recognition test, the table entries refer to the proportion of 'old' responses made to old objects (the hit rate) and the proportion of 'old' responses to new objects (the false-alarm rate). To compare recognition accuracy for possible and impossible objects, we computed a corrected recognition score by subtracting the false-alarm rate from the hit rate. The corrected recognition score for possible objects (0.49) was significantly higher than the corrected recognition score for impossible objects (0.32), ($t(15) = 4.58$, $P < 0.001$). Nearly identical results were obtained in the pilot study using a separate set of subjects and the same test procedures. These findings replicate results from numerous previously published experiments^{2,4,10,29}. The overall levels of 'old' and 'new' responses are also similar to published results from the same experiments. That we replicated both the quantitative and qualitative findings of previous research is important because the demands of PET scanning required that we present old possible, new possible, old impossible, and new impossible objects in separate blocks. This is a substantial departure from the standard practice of intermixing objects from different conditions, but our behavioural data indicate that task performance is indistinguishable from performance under standard experimental conditions.

eral prefrontal cortex (Brodmann areas 10, 44-46). For impossible objects, for which there was less accurate episodic memory (Table 1), the same comparisons revealed no significant blood flow increases. Post hoc comparisons indicate that the right hippocampal/parahippocampal blood flow increases during episodic memory for possible objects were greater than those in the left ($P < 0.05$), and were greater than those in the right during the same comparisons for impossible objects ($P < 0.05$).

Our results are consistent with evidence supporting involvement of the hippocampal formation²¹⁻²⁴ and dorsolateral prefrontal cortex²⁴⁻²⁷ in episodic memory. Failure to detect significant blood flow increases in association with episodic recognition of impossible objects could indicate that recognition of these objects was not sufficiently robust, that the right hippocampal region is less involved in recognition of impossible than of possible objects, or that right hippocampal activation depends on both episodic memory and other factors that are not yet well understood²⁷.

This study and others have activated the hippocampal formation, but several previous memory experiments failed to do so²⁵⁻²⁷. Although the exact reasons for the contrasting results remain elusive^{27,28}, we suspect that the novelty of our objects is relevant. We suggest that the left hippocampal/parahippocampal region participated in some aspect of response to these novel stimuli (such as object encoding or identification of a mismatch between internal expectations and novel stimuli), whereas the right hippocampal/parahippocampal region was involved in episodic retrieval of the objects.

Our study attempted to indicate brain regions that are specifically involved in object decision and recognition tasks that we have previously studied behaviourally, so we presented possible or impossible objects in all conditions. It is unknown whether

simply looking at possible or impossible objects, as opposed to a condition in which non-object stimuli are presented, produces differential blood flow increases for possible and impossible objects. A study using additional controls is necessary to determine how the blood flow changes reported here are related to those involved in passive perception of possible and impossible objects.

Additional significant blood flow changes will be described in a subsequent report. For instance, for both possible and impossible objects, the new-object decision, old-object decision, new recognition and old recognition minus no-decision baseline comparisons all revealed increases in the vicinity of dorsolateral prefrontal cortex (Brodmann areas 10, 44–46), which could be involved in the executive operations associated with making a decision.

Our study provides direct neuroanatomical evidence from the normal human brain that inferior temporal and fusiform gyri are selectively involved in computing global representations of structurally coherent three-dimensional objects, and extends our knowledge of the neural systems involved in episodic memory for visual objects. □

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Inhibition by anandamide of gap junctions and intercellular calcium signalling in striatal astrocytes

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ANANDAMIDE, an endogenous arachidonic acid derivative that is released from neurons and activates cannabinoid receptors¹, may act as a transcellular cannabimimetic messenger in the central nervous system^{2–4}. The biological actions of anandamide and the identity of its target cells are, however, still poorly documented⁵. Here we show that anandamide is a potent inhibitor of gap-junction conductance and dye permeability in striatal astrocytes. This inhibitory effect is specific for anandamide as compared to co-released congeners⁴ or structural analogues, is sensitive to pertussis toxin and to protein-alkylating agents, and is neither mimicked by cannabinoid-receptor agonists nor prevented by a cannabinoid-receptor antagonist. Glutamate released from neurons evokes calcium waves in astrocytes⁶ that propagate via gap junctions^{7–9}, and may, in turn, activate neurons distant from their initiation sites in astrocytes^{10–12}. We find that anandamide blocks the propagation of astrocyte calcium waves generated by either mechanical stimulation or local glutamate application. Thus, by regulating gap-junction permeability, anandamide may control intercellular communication in astrocytes and therefore neuron–glial interactions.

The conductance of gap junctions (GJ) was monitored in pairs of cultured striatal astrocytes from embryonic mice by using the double whole-cell recording (DWCR) method¹³ (Fig. 1*Aa, b*). The junctional conductance, which was 14.4 ± 2.7 nS (mean $\pm$

s.e.m.; $n=26$) in the control, was reduced to 1.1 ± 1.2 nS ($n=14$, in which 8 pairs were completely uncoupled) by $5 \mu\text{M}$ anandamide, but not by the cannabinoid agonist CP55940 ($1 \mu\text{M}$) (20.5 ± 5.3 nS; $n=6$) (Fig. 1*B*). This inhibitory effect of anandamide on GJ permeability was confirmed by using the scrape-loading dye-transfer technique¹⁴. The intercellular diffusion of Lucifer yellow was significantly decreased and even greatly inhibited when the cells were incubated for 10 min with anandamide ($1 \mu\text{M}$ and $5 \mu\text{M}$, respectively) (Fig. 1*Ca–c, D*). The inhibition evoked by anandamide ($5 \mu\text{M}$) was reversible, and comparable to that evoked by the uncoupling agent 18α -glycyrrhetic acid¹⁵ ($10 \mu\text{M}$) (Fig. 1*D*), being already apparent after 2 min exposure (data not shown).

In contrast to anandamide, two synthetic cannabinoid-receptor agonists, Win-55212-2 ($5 \mu\text{M}$) and CP55940 ($1 \mu\text{M}$), had no effect on GJ permeability (Fig. 1*D*), although at the same concentrations these two compounds, as anandamide, inhibited the isoprenaline ($1 \mu\text{M}$)-induced production of cyclic AMP in striatal astrocytes (S. Sagan, unpublished results). Moreover, the antagonist at CB1-type cannabinoid receptors¹⁶, SR141716A ($0.5 \mu\text{M}$; 10 min pretreatment), did not reduce the anandamide response (Fig. 1*D*). Therefore, anandamide seems to modulate GJ permeability in striatal astrocytes through a mechanism distinct from central cannabinoid-receptor activation. When cells were pretreated in the presence of the phosphodiesterase inhibitor IBMX (1 mM) with agents known to increase cAMP concentration (such as isoprenaline or forskolin, at $10 \mu\text{M}$ for 10 min), the effectiveness of anandamide on GJ permeability was not altered, suggesting that a reduction of cAMP level is not involved in this process. In addition, neither the protein-kinase inhibitors H7 (0.1 mM , 30 min) or staurosporine ($1 \mu\text{M}$, 30 min), which have a large range of action, nor the phosphatase inhibitor okadaic acid ($0.1 \mu\text{M}$, 40 min) prevented GJ uncoupling induced by anandamide ($5 \mu\text{M}$, 10 min).

The chemical structure of anandamide (*N*-arachidonoyl ethanolamine) includes the polyunsaturated fatty acyl moiety of arachidonic acid (AA), which inhibits GJ permeability in astrocytes¹⁷. However, several observations suggested that anandamide does not act directly on striatal astrocyte GJ by mimicking the uncoupling effect of exogenous application of AA. First, anandamide was ~ 10 times more potent than AA in uncoupling striatal astrocytes (Fig. 1*D*). Second, pertussis toxin

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(PTX), which ADP-ribosylates and inhibits Gi/ α -proteins, markedly reduced the GJ uncoupling evoked by anandamide (5 μ M), but not that produced by AA (50 μ M) (Fig. 1*Cd, e, E*). This lack of effect of PTX on AA-induced inhibition of GJ permeability was not due to the use of a saturating concentration of AA, because at 10 μ M, when partial uncoupling ($40 \pm 4\%$ of control, $n=3$) occurred, PTX treatment had no significant effect ($47 \pm 10\%$, $n=3$). Furthermore, *N*-ethylmaleimide, an alkylating agent that inhibits G proteins¹⁸, and the histidine alkylating agent 4-bromophenacyl bromide each prevented inhibition by anandamide (5 μ M) but not by AA (50 μ M) (Fig. 1*E*).

The selectivity of anandamide was supported further by experiments with structural analogues. We synthesized four anandamide analogues in which the *N*-arachidonoyl moiety was replaced with moieties of equal chain length (20 carbon atoms) but having a decreasing number of double bonds (3, 2, 1 or 0 instead of 4). At a concentration (5 μ M) at which anandamide induced a potent response on GJ permeability in striatal astrocytes, none of these analogues had any significant effect (Fig. 2*a*, and data not shown). Together with anandamide, a family of five *N*-acyl-ethanolamines are released from stimulated striatal neurons⁴. These congeners were not effective when used at 5 μ M (Fig. 2*a* and data not shown), but two of them, *N*-oleoyl-

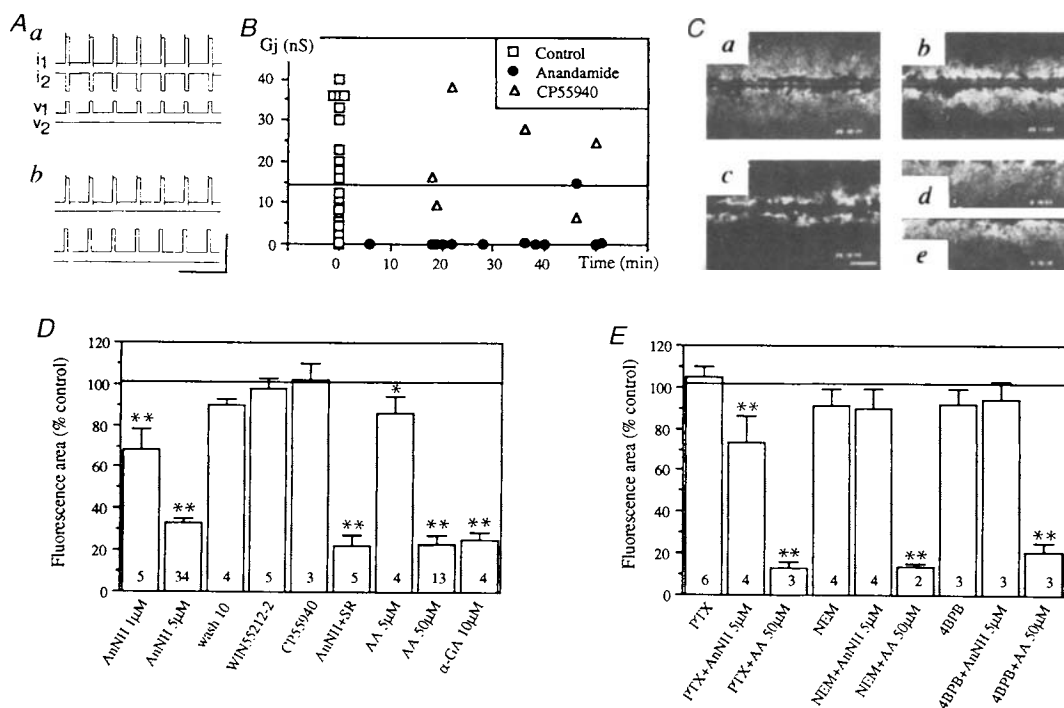


FIG. 1 Inhibition by anandamide of GJ conductance and dye permeability in primary cultures of striatal astrocytes from mouse embryos. **A**, Currents recorded from pairs of astrocytes using the DWCR technique¹³: control junctional current (i_2) was monitored when a voltage difference ($V_1 - V_2$) was applied between the two cells from a -70 mV holding potential (*a*). In the presence of 5 μ M anandamide (*b*), no junctional current was detected in another pair of cells from the same dish (calibrations: 2, 0.5 nA and 2, 0.2 nA, for i_1 and i_2 in *a* and *b* respectively, 200 mV for V_1 and V_2 , 2 s). **B**, Effects of anandamide (5 μ M) and CP55940 (1 μ M) on junctional conductance (G_j , ordinate). G_j was measured 1–2 min after establishment of the DWCR by applying trans-junctional voltage pulses, ranging from $+50$ mV to $+100$ mV, and then plotted as a function of time (abscissa). One or two G_j values were measured in each dish; all these control points were arbitrarily plotted at time 0 when either anandamide or CP55940 were added to the bath. The horizontal line represents the averaged value (14.4 nS) of the controls. **C**, Fluorescence photomicrographs of scrape-loading experiments: *a*, control, 10 min after loading, Lucifer yellow (LY) diffused widely throughout the confluent layer of astrocytes; *b*, 1 μ M anandamide significantly reduced dye diffusion; *c*, 5 μ M anandamide blocked dye spread; *d*, PTX treatment had no significant effect (1 μ g ml⁻¹, 24 h); *e*, the inhibitory effect of 5 μ M anandamide was reduced by PTX treatment (*d* and *e* represent half of photomicrographs; calibration bar, 250 μ m). **D**, **E**, averaged values (mean $\pm$ s.e.m.) of the surface area stained with Lucifer yellow, measured under various conditions and expressed as percentage of the internal controls. Drugs were applied for 10 min before scrape-loading and used at the concentrations indicated in the text. Pretreatments with NEM (50 μ M) and 4BPB (10 μ M) were made 10 and 30 min before anandamide or AA application, respectively. The number of independent experiments performed are indicated inside the columns. Statistical analysis: one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test. Significance was established at

$P < 0.05$ (*) and $P < 0.01$ (**). Horizontal line indicates the level of fluorescence area determined in control conditions. Abbreviations: AnNH, anandamide; PTX, pertussis toxin; α -GA, 18 α -glycyrrhetic acid; NEM, *N*-ethylmaleimide; 4BPB, 4-bromophenacyl bromide. **METHODS**. Primary cultures of astrocytes were prepared as described^{17,26}. Selected brain structures were removed from 16-day-old Swiss mouse embryos. The culture medium, a mixture of EM medium and F12 nutrient (1:1) supplemented with 10% NU serum, was changed weekly (serum was omitted 4 days before experiments). Experiments were done at room temperature on 18–24-day-old cultures, in which up to 95% of the cells were identified as astrocytes by using an antibody against the glial fibrillary acidic protein (GFAP). External solution contained (in mM): NaCl 140, KCl 5.5, CaCl₂ 1.8, MgCl₂ 1, glucose 25 and HEPES 10, pH 7.3. Junctional conductance was monitored using the DWCR technique¹³ performed on pairs of astrocytes prepared as described²⁶, with a recording pipette solution containing (in mM): KCl 140, MgCl₂ 1, EGTA 5 and HEPES 10, pH 7.3. GJ permeability was determined using the scrape-loading technique¹⁴. Cell loading was achieved with a blade in the external solution (without calcium) containing 0.1% LY; 1 min later, cells were washed several times with the standard external solution. When a cocktail of LY (*M*, 457) and rhodamine-dextran (*M*, 10,000) was used, the localization of the second dye, to which GJs are impermeable, was restricted to the initially loaded cells, whereas LY diffused to neighbours (*C, a*). GJ permeability was assessed 9 min after scraping by taking photomicrographs of five adjacent hemifields. Analysis was carried out with an image analyser system (Imstar software), measuring the fluorescence area from digitized images¹⁷. Quantification was then achieved by expressing the measured area as a percentage of its internal control. Applications of vehicles (methanol, 1/2,000; ethanol 1/2,000; dimethylsulphoxide, 1/20,000) alone had no effect on GJ permeability.

TABLE 1 Effects of different agents on the propagation of calcium waves in striatal astrocytes

Experimental conditions		Number of cells in the field (no. of experiments)	Number of responding cells (%)	Ratio (F405/F480) of stimulated cell (no. of cells)	Ratio (F405/F480) of the cells of the first row (no. of cells)
Mechanical stimulation	Control	34 ± 1 (25)	28 ± 1 (82%)	1.17 ± 0.04 (25)	0.76 ± 0.07 (137)
	Anandamide 0.01 µM	33 ± 1 (14)	29 ± 1.4 (88%)	1.17 ± 0.03 (14)	0.71 ± 0.03 (96)
	0.1 µM	36 ± 1 (13)	30 ± 1 (83%)	1.24 ± 0.08 (13)	0.86 ± 0.03 (92)
	0.5 µM	30 ± 2 (4)	11 ± 1 (37%)	1.03 ± 0.14 (4)	0.17 ± 0.02 (11)
	1 µM	30 ± 4 (4)	5 ± 1 (15%)	0.96 ± 0.06 (4)	0.16 ± 0.01 (17)
	5 µM	32 ± 2 (15)	4 ± 0.8 (13%)	1.03 ± 0.05 (15)	0.27 ± 0.03 (35)
	Win 55212-2 5 µM	34 ± 2 (3)	25 ± 1.8 (74%)	1.39 ± 0.02 (3)	0.58 ± 0.06 (19)
	C18:1 30 µM	31 ± 2 (5)	24 ± 1 (77%)	1.09 ± 0.11 (5)	0.56 ± 0.06 (30)
Local application of glutamate (100 µM)	Arachidonic acid 10 µM	35 ± 2 (15)	4 ± 1 (11%)	1.04 ± 0.05 (15)	0.25 ± 0.04 (23)
	18α-glycyrrhetinic acid 10 µM	30 ± 3 (4)	2 ± 1 (7%)	1.06 ± 0.12 (4)	0.16 ± 0.08 (4)
	Control	30 ± 1 (11)	11 ± 0.8 (37%)	0.43 ± 0.04 (11)	0.29 ± 0.02 (36)
	Anandamide 5 µM	29 ± 1 (20)	2 ± 0.4 (6%)	0.34 ± 0.03 (20)	0.13 ± 0.02 (21)

Averaged values given in the second column represent the number of cells in which a rise in $[Ca^{2+}]_i$ was recorded (responding cells); percentages are expressed versus the number of total cells in the microscopic field. Velocity of intercellular calcium waves, generated either by mechanical stimulation or local glutamate application, were calculated from control experiments and were $17 \pm 1 \mu m s^{-1}$ and $15 \pm 1 \mu m s^{-1}$, respectively. Results are expressed as mean $\pm$ s.e.m.

ethanolamine and *N*- γ -linolenoyl-ethanolamine, had a significant, albeit partial, effect at 30 μM and 10 μM , respectively; these concentrations take into account their ratio of co-release with anandamide⁴ (Fig. 2a).

We then determined whether the ability of anandamide to inhibit GJ dye permeability was different in astrocyte cultures prepared from various brain structures. In contrast to the marked uncoupling observed in striatal astrocytes, anandamide (5 μM) only partially affected GJ permeability in astrocytes from other brain structures. No such regional specificity was observed with AA used at concentrations that produce either partial (10 μM) or complete (50 μM) uncoupling in striatal astrocytes (Fig. 2b).

In astrocytes, GJs provide a pathway for the propagation of intercellular calcium waves^{7,9}, which in turn may be important in astrocyte-neuron interactions^{10-12,19}. Striatal astrocytes were loaded with Indo-1-AM to determine the effect of anandamide on calcium waves. Mechanical stimulation of individual astrocytes⁷ induced a rapid rise in intracellular free calcium level ($[Ca^{2+}]_i$),

which spread to distant astrocytes (Fig. 3a). Anandamide (5 μM) blocked these calcium waves as in this case only direct neighbours of the stimulated cell showed slight rises in $[Ca^{2+}]_i$ (Fig. 3b and Table 1). Such an effect was already detected with a concentration of anandamide as low as 0.5 μM (Table 1), similar to that required to inhibit the isoprenaline-induced production of cAMP in striatal astrocytes (S. Sagan, unpublished results) and to obtain half-maximal activation of cannabinoid receptors in other intact cells²⁰. Owing to the rapid astrocytic uptake and hydrolytic degradation of anandamide^{4,21}, the effective concentration of this compound may have even been lower than 0.5 μM . Although lower concentrations of anandamide (0.1 and 0.01 μM) had no effect, calcium waves were also blocked by AA (10 μM) and 18 α -glycyrrhetinic acid (10 μM) but not by *N*-oleoyl-ethanolamine (30 μM) or Win 55212-2 (5 μM) (Table 1). Calcium waves were also elicited by applying the excitatory neurotransmitter, glutamate, onto individual astrocytes with a pressure-operated micropipette (Fig. 3c). These waves, which propagated typically along circuitous

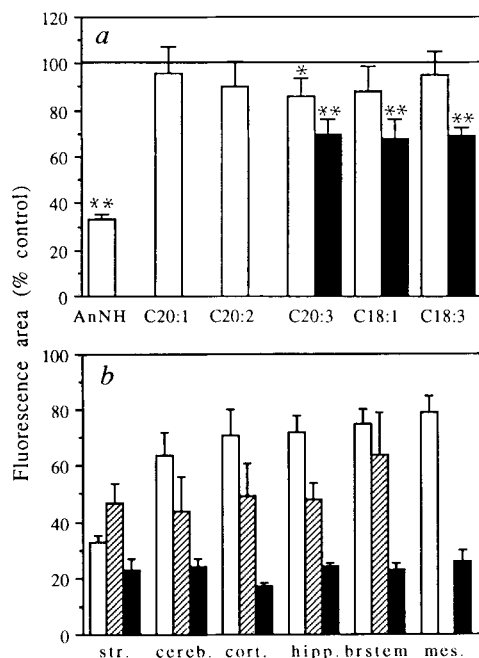
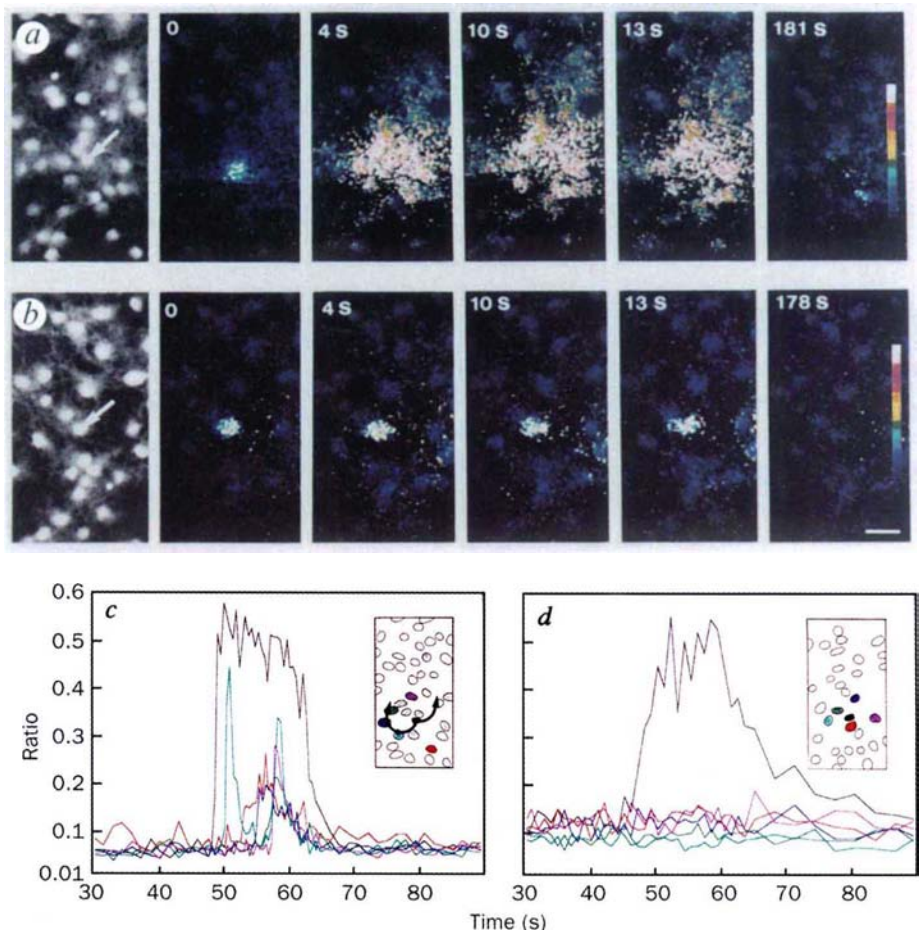


FIG. 2 Structural and regional specificity of anandamide-induced inhibition of astrocyte GJ permeability. **A**, Effects of anandamide (*N*-arachidonyl-ethanolamine, AnNH), *N*-eicosamonoenoylethanolamine (C20:1), *N*-eicosadienoylethanolamine (C20:2), *N*-eicosatrienoylethanolamine (C20:3), *N*-oleoyl-ethanolamine (C18:1) and *N*- γ -linolenoyl-ethanolamine (C18:3) on GJ permeability in striatal astrocytes. Concentrations used were 5 μM (empty columns) and 10 μM (for C18:3 and C20:3) or 30 μM (for C18:1) (filled columns). **B**, Effects of 5 μM anandamide (empty columns), 10 μM (hatched columns) and 50 μM AA (filled columns) on GJ permeability of astrocyte cultured from striatum (str.), cerebellum (cereb.), cortex (cort.), hippocampus (hipp.), brainstem (br. stem), and mesencephalon (mes.). Drugs were applied for 10 min before scrape-loading. The number of independent experiments shown in **a** and **b** varied from 3 to 34. Statistical analysis: in **a**, significance was established at $P < 0.01$ (*) and $P < 0.0001$ (**) with Student's *t*-test; in **b**, using one-way ANOVA followed by *post hoc* Student-Newman-Keuls's multiple comparisons, test significance was established at $P < 0.001$ for anandamide in striatum versus other structures, but no significance was found for all structures with AA at 10 μM and 50 μM ($P > 0.05$).

METHODS. Primary cultures of astrocytes were prepared and used as described in Fig. 1 legend, except for the mesencephalon and the cerebellum, which were prepared from 14-day-old embryos and newborn animals, respectively. Anandamide and its analogues were synthesized⁴ and purified by high-performance liquid chromatography as described². All products were kept in methanol (10 mM) at $-80^\circ C$, and diluted in standard ionic solutions immediately before the experiments.

FIG. 3 Blockage by anandamide of propagation of calcium waves in primary cultures of rat striatal astrocytes. Changes in $[Ca^{2+}]_i$ were monitored by calcium imaging. Mechanical stimulation of individual astrocytes (arrows), carried out in the absence (a) or presence (b) of anandamide (5 μ M). Left, fluorescent images ($\lambda_{\text{emission}} = 480$ nm) of two fields of astrocytes loaded with Indo1-AM; right, sequential pseudocolour representations of $[Ca^{2+}]_i$, expressed as the ratio of indo1-AM emissions at 405 and 480 nm due to excitation at 355 nm. Calcium increase initiated in the stimulated cell rapidly propagated in all directions to most cells present in the microscopic field ($t = 13$ s). In the control experiment (a) the averaged calcium basal level of the cells in the investigated field was 0.10 ± 0.01 ($n = 37$ cells) before mechanical stimulation, and the peak of $[Ca^{2+}]_i$ changes following mechanical stimulation varied from 0.18 to 1.46 ($n = 34$ cells). In the presence of anandamide (b), basal calcium was 0.11 ± 0.01 nM ($n = 27$ cells) and the mean peak of $[Ca^{2+}]_i$ change induced in the stimulated cell and the adjacent cells varied from 0.06 to 1.22 nM ($n = 6$ cells). Pseudocolour scale bars refer to ratios from 0.01 to 1.50, which corresponded to estimated $[Ca^{2+}]_i$ values of 10 nM to 2,000 nM, respectively (calibration bar, 25 μ m). Stimulation of individual astrocytes by pressure application of glutamate (100 μ M, ~ 276 kPa, 20 ms) either in the absence (c) or presence (d) of anandamide (5 μ M). Insets: schematic drawings of astrocyte cell bodies in the inspected microscopic fields. Black, astrocytes receiving the glutamate puffs; colour, cells in which fluorescence was plotted; grey, additional responding cells. Arrows indicate the paths of two separate calcium waves evoked by glutamate application.



METHODS. Striatal astrocytes from 18-day-old rat embryos were maintained in culture as described for Fig. 1, except that they were plated on glass coverslips coated with poly-L-ornithine and laminin²⁷. Intracellular calcium levels were measured by dual emission microfluorimetry using the fluorescent dye Indo1-AM²⁷. $[Ca^{2+}]_i$ was calculated from the fluorescence ratio (measured at 400–410 nm and 470–480 nm)

according to Grynkiewicz's equation²⁸, with a K_d of 250 nM for the Indo1-AM. Images were acquired every 1–3 s with an image-processing system (Hamamatsu Argus 50) and an inverted microscope (Nikon Diaphot) and then recorded on an optical disk memory (Laser Magnetic Storage International Company). Experiments were carried out at room temperature in a solution containing (in mM): NaCl 145, KCl 5.5, $CaCl_2$ 1.1, $MgCl_2$ 0.9, glucose 12 and HEPES 20, pH 7.3.

paths⁸, were also blocked by anandamide (5 μ M) (Fig. 3d and Table 1).

Our results show that anandamide inhibits GJ conductance and dye permeability, and blocks GJ-mediated propagation of calcium signalling in striatal astrocytes. Because of its brain-structure specificity, structural selectivity, sensitivity to G-protein inhibitors and to mild protein-alkylating agents, the anandamide GJ inhibition could be mediated through a specific receptor. Such a putative receptor may be distinct from any of the two known cannabinoid receptor subtypes⁵, as suggested by the lack of effect of either cannabinoid-receptor agonists or CB-1 antagonist. Further supporting this possibility, recent studies have indicated that anandamide, a partial agonist at cannabinoid receptors¹⁸, may exert several pharmacological actions distinct from those of plant-derived or synthetic cannabinoid drugs²².

Glutamate released from nerve terminals evokes calcium waves in adjacent astrocytes⁷. In turn, these waves propagate through the astrocyte network²³ and trigger responses in neurons embedded within it^{10–12}. Our findings suggest that this long-range signalling loop between neurons and astrocytes may be turned off by anandamide. Thus anandamide, a transcellular messenger produced and released from neurons but not from astrocytes^{2,4}, may modulate synaptic function not only locally by controlling neuronal transmitter release through the regulation of calcium channels¹⁸, but also distally by preventing the recruitment of

subpopulations of astrocytes and the subsequent activation of contacting groups of neurons. It remains to be determined whether such a distal action of anandamide occurs as well *in vivo* in the striatum, where its astrocyte-uncoupling effect is most prominent and the existence of functional compartments of neuronal groups receiving somatotopic glutamatergic cortical afferents has been demonstrated^{24,25}. □

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An IL-12-based vaccination method for preventing fibrosis induced by schistosome infection

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THE harmful fibrosis which often occurs in the context of infectious disease involves the excessive deposition of connective tissue matrix, particularly collagen, and is mostly resistant to pharmacological and immunological intervention¹. In schistosomiasis, fibrosis is associated with the granulomatous response to parasite eggs trapped in the liver². We have previously shown that interleukin (IL)-12 administered peritoneally with eggs prevents subsequent pulmonary granuloma formation on intravenous challenge with eggs³. Here we show that sensitization with eggs plus IL-12 partly inhibits granuloma formation and dramatically reduces the tissue fibrosis induced by natural infection with *Schistosoma mansoni* worms. These results are an example of a vaccine against parasites which acts by preventing pathology rather than infection. IL-12 is known to favour the priming of Th1 rather than Th2 cells, and the effects on fibrosis are accompanied by replacement of the Th2-dominated pattern of cytokine expression characteristic of *S. mansoni* infection with one dominated by Th1 cytokines. Elevated Th2 cytokine expression and fibrosis are common manifestations of a wide variety of infectious diseases and atopic disorders which might be ameliorated by vaccination with antigen and IL-12.

Mice of the C57BL/6 strain were inoculated with *S. mansoni* eggs, either with or without IL-12, and then exposed to percutaneous infection by *S. mansoni* larvae (cercariae). Egg/IL-12 sensitization did not significantly reduce the number of worms or eggs recovered early in infection at either 8 or 12 weeks (Table 1). Nevertheless, sensitization with eggs/IL-12 had pronounced effects on host pathology. Granulomas were evident in the egg/IL-12 group, but the lesions were significantly smaller and contained fewer eosinophils than those observed in infected unsensitized mice (Table 1). Sensitization with eggs alone resulted in a smaller decrease in granuloma size, comparable to that seen in previous studies⁴. The most striking effect of egg/IL-12 sensitization was the inhibition of egg-induced hepatic fibrosis, as illustrated by tissue sections stained for collagen (Fig. 1). Compared with control mice at 8 and 12 weeks, mice sensitized with egg/IL-12 showed reductions in hepatic hydroxyproline (a chemical measure of collagen content) of 58.0% and 72.2%, respectively, and reductions of 19.5% and 35.5% were observed in the egg/saline group (Table 1). When levels of

messenger RNA for type I and type III collagen are compared (Fig. 2), the differences between the three groups of mice are greatest at 8 weeks, during the initial phase of synthesis of these proteins. Sensitization with eggs/IL-12 caused a nearly tenfold reduction in the levels of induced expression of liver type I and type III mRNAs relative to that seen in unsensitized mice. A 2.5-fold and 4-fold reduction was seen in the same mRNAs in the egg/saline group. The observed sensitization-dependent decrease in tissue pathology was not accompanied by increases in necrosis within granulomas.

Previous studies have demonstrated an influence of Th2 cytokines on hepatic granuloma formation and fibrosis^{5,6}, and a suppressive effect of IL-12 on egg-induced Th2 cytokine expression^{3,7}. We therefore examined Th2- and Th1-associated cytokine mRNA profiles in the liver at 8 and 12 weeks. As expected^{8,9}, there was a marked elevation in the Th2-associated

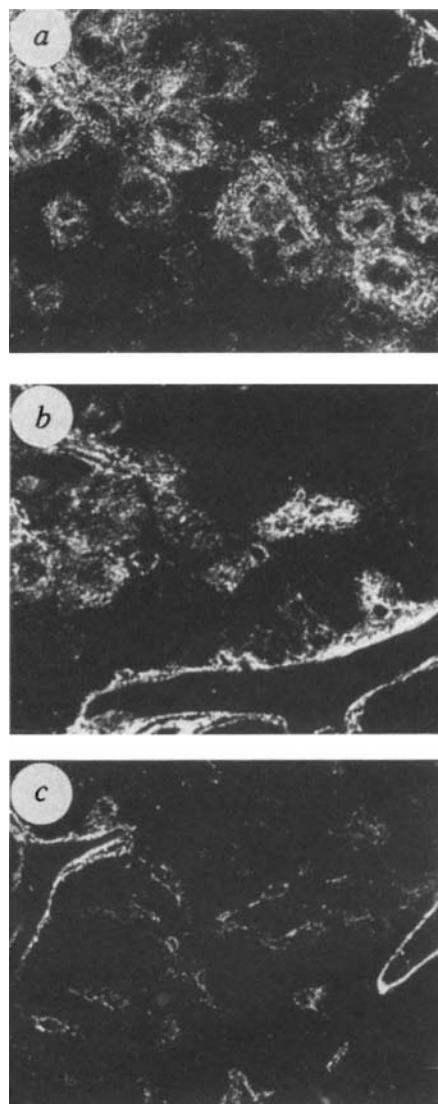


FIG. 1 Hepatic collagen production is reduced in infected mice sensitized with eggs/IL-12. Liver sections were obtained 12 weeks after challenge infection. Unsensitized (a), egg/saline-sensitized (b), egg/IL-12-sensitized (c) and infected mice that contained nearly identical tissue egg burdens were stained with picosirius red and photographed using polarized light, and the areas shown are representative for each liver. Birefringent areas indicate collagen (magnification, $\times 34$). Liver sections from egg/IL-12-immunized mice showed only slight granuloma and portal tract-associated collagen, in comparison with non-vaccinated animals.

TABLE 1 Parasitological measurements in sensitized mice

Week post-infection	Group	Parasite recovery		Granuloma formation			Fibrosis	
		Worm pairs	Eggs per worm pair ($\times 1000$)	Volume ($10^{-3} \times \text{mm}^3$)	Eosinophil (%) [*]	Necrosis (0–4) [†]	Hydroxyproline ($\mu\text{mol per g liver}$)	Hydroxyproline ($\mu\text{mol per } 10^4 \text{ eggs}$)
8	Control infected	3.4 $\pm$ 0.68	6.59 $\pm$ 0.74	15.9 $\pm$ 1.01	57 $\pm$ 3.3	2.2	5.70 $\pm$ 0.3	4.10 $\pm$ 0.3
	Egg/saline	3.2 $\pm$ 0.66	7.91 $\pm$ 1.96	12.2 $\pm$ 0.55 [‡]	48 $\pm$ 3.3	1.3	4.10 $\pm$ 0.3 [‡]	3.30 $\pm$ 0.4 [‡]
	Egg/IL-12	3.44 $\pm$ 0.75	5.70 $\pm$ 1.12	9.92 $\pm$ 1.68 [‡]	22 $\pm$ 3.2 ^{‡§}	0.5	2.90 $\pm$ 0.3 ^{‡§}	1.70 $\pm$ 0.1 ^{‡§}
12	Control infected	5.55 $\pm$ 0.64	14.4 $\pm$ 1.29	14.6 $\pm$ 0.84	43 $\pm$ 3.0	1.1	10.1 $\pm$ 0.9	5.4 $\pm$ 0.6
	Egg/saline	4.87 $\pm$ 0.57	13.0 $\pm$ 1.06	9.58 $\pm$ 0.69 [‡]	44 $\pm$ 2.0	0.5	6.20 $\pm$ 0.2 [‡]	3.5 $\pm$ 0.3 [‡]
	Egg/IL-12	3.75 $\pm$ 0.84	12.8 $\pm$ 1.30	9.40 $\pm$ 1.15 [‡]	31 $\pm$ 5.4 [§]	0.8	2.8 $\pm$ 0.4 ^{‡§}	1.5 $\pm$ 0.2 ^{‡§}

Unsensitized, egg-sensitized and egg/IL-12-sensitized mice were killed either 8 or 12 weeks after infection. Female C57BL/6 mice were immunized 3 times, separated by 3-week intervals, by intraperitoneal injection of 5000 *S. mansoni* eggs isolated from the livers of 7-week-infected mice. Animals were also injected intraperitoneally with IL-12 (0.25 μg per dose) or saline on days 0, 1, 2 and 3 after each egg exposure. Mice were infected with 25 *S. mansoni* cercariae and worms were later recovered by perfusion²⁵. Eggs were counted after digestion of liver and intestines in 4% KOH. Granuloma diameters (about 30 per mouse) were measured using an ocular micrometer, and the percentage eosinophils in measured granulomas was estimated in sections stained with Litt's modification of the Dominici stain. Mean granuloma volumes were calculated for each mouse from the volumes of individual granulomas. Hepatic hydroxyproline levels were determined as previously described²⁶. The percentage change noted in the text is based on hydroxyproline levels adjusted to $\mu\text{mol per } 10,000 \text{ eggs}$. Differences were highly significant by covariance analysis (B versus C, $P < 0.01$, $P < 0.002$ on weeks 8 and 12, respectively). Values represent the means $\pm$ s.e. of 8–10 mice per group.

^{*} Percentage eosinophils within granulomas.

[†] Central necrosis within granulomas was scored from absent (0) to most severe (4).

[‡] Group is significantly different from group A at that time point ($P < 0.05$) by analysis of covariance and Student's *t*-test.

[§] Group is significantly different from group B at that time point ($P < 0.05$). We observed no effect on pathological parameters when IL-12 was administered alone (without antigen) before infection (data not shown). The effect of egg/IL-12 sensitization on more chronic (>12 weeks) infection was not examined in this study. All experiments have been repeated with similar results. Similar results were also obtained in a pilot experiment in which soluble egg antigen (SEA) was substituted for parasite eggs as the immunogen.

cytokine mRNAs (IL-4, IL-5, IL-13 and IL-10), and an increase in interferon (IFN)- γ mRNA, in the livers of unsensitized mice 8 weeks after infection (Fig. 2), but only small increases in IL-2, IL-12(p40) and tumour-necrosis factor (TNF)- α were observed. In contrast, mice sensitized with egg/IL-12 showed markedly elevated levels of the Th1-associated IL-2, IL-12(p40) and TNF- α mRNAs, whereas hepatic IL-5 and IL-13 mRNA expression was almost completely inhibited, although IL-10 expression was not suppressed (a result consistent with related studies³). Egg/saline sensitization did not increase the Th2 cytokine response in the liver, as might have been anticipated; IL-5 and IL-13 mRNA levels were actually reduced. We speculate that the reduction in both fibrosis and cytokine expression seen in mice sensitized with egg/saline is due to an accelerated progression to the 'downmodulated' state⁴ found at later stages of infection.

Downmodulation of the Th2 response in the spleen and mesenteric lymph nodes is seen by 18 weeks after infection, even in unsensitized mice, although a suppressive effect of egg/IL-12 sensitization on Th2 cytokines remains evident at this stage (Fig. 3). At the earlier time of 8 and 12 weeks, a Th1- rather than Th2-dominated T-cell response appears as a consequence of egg/IL-12 sensitization (Fig. 3), adding further support to the association of reduced hepatic fibrosis with suppressed Th2- and increased Th1-associated cytokine production.

Because transforming growth factor (TGF)- β can directly trigger the proliferation of fibroblasts and stimulate the production of type I and type III collagens^{1,10,11}, we also examined the production of this cytokine (Fig. 2). TGF- β expression was downmodulated in sensitized mice, but at 8 weeks its expression was equally decreased in mice vaccinated with eggs with or without IL-12. Thus regulation of collagen synthesis by TGF- β cannot provide more than a partial explanation for the reduction in fibrosis. The enhanced production of IFN- γ and TNF- α induced by prior sensitization with eggs and IL-12 may also be involved. These two cytokines have been reported to suppress collagen synthesis^{1,12}, although in some circumstances TNF- α can induce fibrosis^{13,14}.

An antipathology vaccine approach has been previously proposed^{4,15}, but is demonstrated successfully for the first time

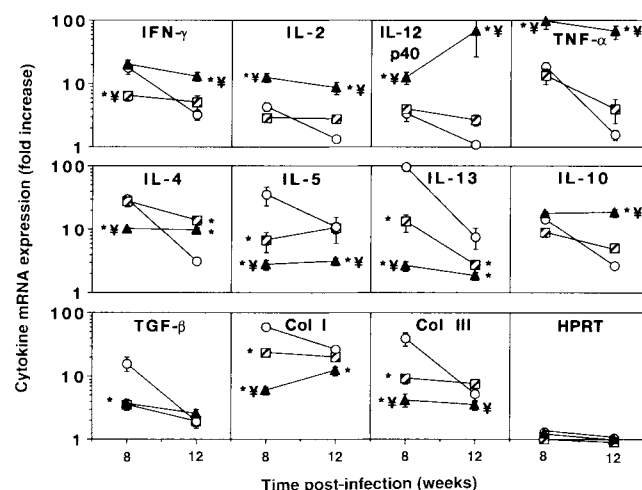


FIG. 2 Alterations in infection-induced hepatic cytokine mRNA expression in mice sensitized with eggs/IL-12. Unsensitized (open circles), egg/saline-sensitized (hatched squares) and egg/IL-12-sensitized (filled triangles) mice were assayed individually for changes in cytokine mRNA expression 8 and 12 weeks post-challenge infection. Values represent the mean 'fold' increase (as a multiple of control value) ($\pm$ s.e.) over uninfected control liver mRNA expression for 8–10 mice per group at each time point.

METHODS. RT-PCR detection of cytokine mRNA was performed as previously described⁸. The amount of PCR product was determined by comparison of signal density with that of standard curves. Increases were based on changes over baseline levels observed in uninfected mouse liver (assigned an arbitrary value of 1). Hypoxanthine phosphoribosyl transferase (HPRT), a housekeeping gene, was used as an internal standard to confirm that equal quantities of mRNA were utilized in the RT-PCR. The change in mRNA expression for several Th1 (IFN- γ , IL-2, IL-12 (p40 subunit)) and Th2-associated cytokines (IL-4, IL-5, IL-13, IL-10) was determined. Asterisks indicate that the group is significantly different from unsensitized controls (open circles) at that time point ($P < 0.05$) by Student's *t*-test, crossed Y symbols indicate that the egg/saline-sensitized and egg/IL-12-sensitized mice are significantly different at that time point ($P < 0.05$).

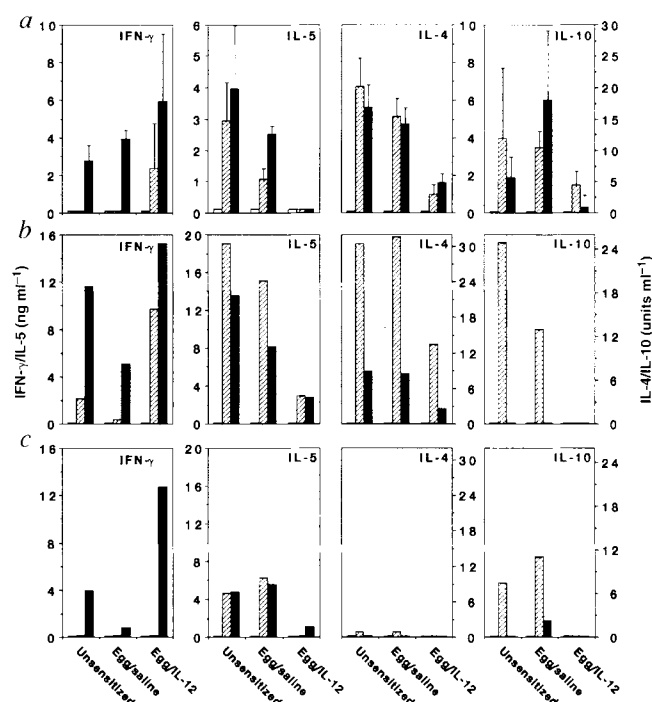


FIG. 3 Sensitization with eggs/IL-12 converts the helminth-induced Th2 response to a Th1-dominated pattern. Spleen (a) and mesenteric lymph node cells (b, c) from unsensitized, egg/saline-sensitized, and egg/IL-12-sensitized mice at 8 (a, b) and 18 (c) weeks post-infection were assayed by ELISA²⁷ for the production of IFN- γ , IL-5, IL-4 and IL-10 following *in vitro* stimulation with medium (white bars), 20 $\mu\text{g ml}^{-1}$ SEA (hatched bars) or 5 $\mu\text{g ml}^{-1}$ concanavalin A (black bars). METHODS. The spleens and mesenteric lymph nodes were isolated from mice sensitized and infected as described in Table 1 legend. Lymph-node cells were pooled from 3–8 animals per group. Spleens were processed individually and values are reported either in ng ml^{-1} (IFN- γ and IL-5) or in $\text{units ml}^{-1} \pm \text{s.d.}$ (IL-4 and IL-10). Cytokine levels were calculated using standard curves constructed using r-murine cytokines. As seen at the mRNA level, egg/saline-sensitized and unsensitized spleen and lymph-node cells produced substantial amounts of IL-4, IL-5 and IL-10 in response to both SEA and mitogen (concanavalin A) stimulation, but little or no IFN- γ was detected in response to SEA. In contrast, spleen and lymph-node cells from mice sensitized with eggs/IL-12 produced little if any Th2 cytokines, although significant levels of IFN- γ were made in response to both SEA and mitogen at 8 weeks (a, b) and to mitogen alone at week 18 (c). Significant Th2-associated cytokine expression was not observed even at late (chronic) stages of infection (c) in egg/IL-12-sensitized mice and, as expected, cytokine production in general was downmodulated when compared with that seen 8 weeks post-infection. The spleen and mesenteric lymph-node data were obtained in separate experiments to confirm reproducibility and similarity in response between different tissues.

here. In schistosomiasis, the major vaccine strategy so far has been immunization against invading larval stages to prevent or reduce infection^{16, 18}, but only limited protection has been achieved¹⁹. Because the disease is largely caused by the host response to eggs, rather than the infecting worms themselves, an antipathology vaccine might prove more effective.

IL-12 has been shown in a variety of experimental models to be a potent immunomodulator^{20–22} and, as originally demonstrated for *Leishmania major*²³, its addition to vaccines can greatly enhance the level of protection achieved. IL-12 has been shown to increase the protective immunity provided by an attenuated larval schistosome vaccine²⁴. Thus, by using the appropriate combination of larval plus egg antigens, it might be possible to design an IL-12-based vaccine which elicits partial protection while reducing the egg-induced pathology caused by worms that escape the protective response. □

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An IRF-1-dependent pathway of DNA damage-induced apoptosis in mitogen-activated T lymphocytes

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LYMPHOCYTES are particularly susceptible to DNA damage-induced apoptosis, a response which may serve as a form of 'altruistic suicide' to counter their intrinsic high potential for mutation and clonal expansion¹. The tumour suppressor p53 has been shown to regulate this type of apoptosis in thymocytes^{2,3}, but an as yet unknown, p53-independent pathway(s) appears to mediate the same event in mitogen-activated mature T lymphocytes⁴. Here we show that DNA damage-induced apoptosis in these T lymphocytes is dependent on the antioncogenic transcription factor interferon regulatory factor (IRF)-1 (refs 5–7). Thus two different anti-oncogenic transcription factors, p53 and IRF-1, are required for distinct apoptotic pathways in T lymphocytes. We also show that mitogen induction of the interleukin-1 β converting enzyme (ICE) gene^{8–10},

a mammalian homologue of the *Caenorhabditis elegans* cell death gene *ced-3*, is IRF-1-dependent. Ectopic overexpression of IRF-1 results in the activation of the endogenous gene for ICE and enhances the sensitivity of cells to radiation-induced apoptosis.

IRF-1 was originally identified as a transcription factor that binds to DNA sequence elements (IRF-Es) found in the promoters of type I interferon (IFN) and IFN-inducible genes^{5,11,12}. As well as regulating the IFN system, IRF-1 manifests tumour-suppressive activities^{6,7}, and its inactivation may be linked to the development of human haematopoietic malignancies^{13,14}. IRF-1 is also required for the induction of apoptosis following DNA damage or culture in low serum in fibroblasts carrying an activated c-Ha-ras gene⁷.

To investigate the possible role of IRF-1 in radiation-induced apoptosis of T lymphocytes, splenocytes from mice with a null

mutation in the *IRF-1* gene (*IRF-1*^{-/-} mice) and those from wild-type mice were mitogenically activated by culturing with concanavalin A (conA) for 3 days and were then exposed to γ -irradiation at 10 Gy in the presence of interleukin (IL)-2. Radiation-induced death was significantly reduced in cells from *IRF-1*^{-/-} mice compared with wild-type cells (Fig. 1a, c). Similar results were obtained with cells treated with the DNA-damaging chemotherapeutic agents adriamycin and etoposide (Fig. 1a). Nuclear morphology (Fig. 1b) and flow cytometric analysis of DNA content (data not shown) indicated that cells were undergoing apoptosis. In contrast, radiation-induced apoptosis of thymocytes was not affected in cells from *IRF-1*^{-/-} mice although, as reported previously^{2,4}, apoptosis was dependent on p53 (Fig. 1c).

IRF-1 gene expression is strongly induced in conA-stimulated splenocytes⁵ (Fig. 2a). To examine the possibility that IRF-1 might regulate known genes involved in apoptosis, we examined the messenger RNA expression of *Ice*, *Ich-1*, *bcl-2* and *bax*. *Ice*, a mammalian homologue of the *Caenorhabditis elegans* cell death gene *ced-3*, has been shown to act as a mammalian cell death gene¹⁵. The *Ich-1* gene, also called *Nedd-2*, is homologous to *Ice* and encodes both a positive and negative regulator of cell death¹⁶. The gene *bcl-2* and the structurally related *bax* encode a negative and positive regulator of apoptosis, respectively¹⁷⁻¹⁹. Of these mRNAs only *Ice* was dramatically affected by the loss of IRF-1 (Fig. 2a). In wild-type splenocytes the *Ice* gene was induced following stimulation with conA, consistent with results previously obtained with activated peripheral human T

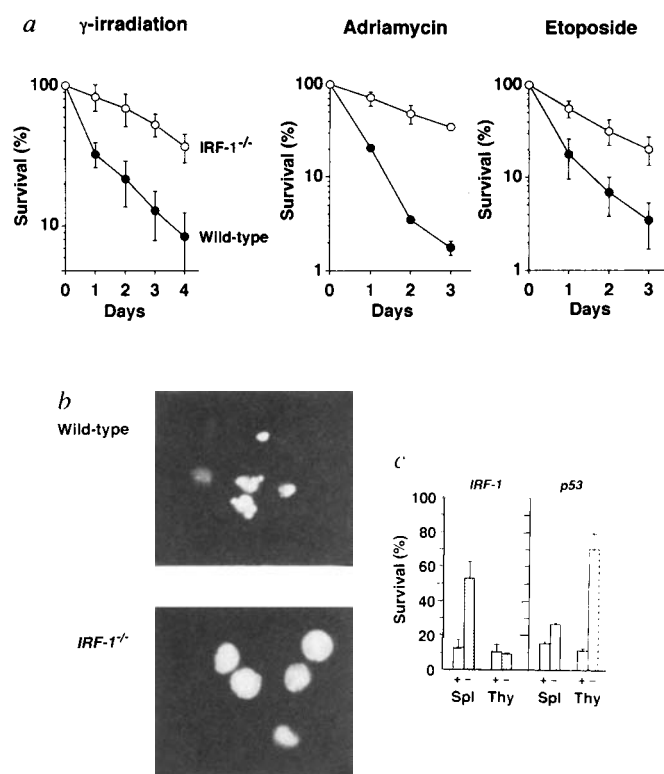


FIG. 1 DNA damage-induced apoptosis in conA-stimulated splenocytes depends on IRF-1. **a**, Survival of conA-stimulated wild-type (filled circles) or *IRF-1*^{-/-} (open circles) splenocytes following γ -irradiation, or exposure to adriamycin or etoposide. **b**, Nuclear morphology of splenocytes one day after γ -irradiation. **c**, Survival of wild-type, *p53*^{-/-} and *IRF-1*^{-/-} thymocytes (Thy) one day after, and conA-stimulated splenocytes (Spl) three days after, γ -irradiation. + and - refer to wild-type and mutant cells, respectively; vertical bars represent standard deviations from three independent samples.

METHODS. Splenocytes and thymocytes were isolated from *IRF-1*^{-/-} mice²⁸ and their wild-type relatives, or *p53*^{-/-} mice²⁹ and their wild-type relatives, all aged 5–8 weeks, and cultured in RPMI 1640 plus 10% fetal calf serum. Splenocytes were cultured in the presence of 5 $\mu\text{g ml}^{-1}$ conA (Pharmacia) for 3 days and then washed, replated in the presence of 100 U ml^{-1} recombinant murine IL-2 at a density of 1×10^6 cells ml^{-1} and irradiated (10 Gy) or treated with 0.5 $\mu\text{g ml}^{-1}$ adriamycin (Kyowa Hakko) or 10 $\mu\text{g ml}^{-1}$ etoposide (Sigma). Survival of cells was determined by trypan blue exclusion and calculated as percentage viable cells relative to number at start of trial. Cells from three different mice of each genotype were examined in each trial, and results were reproducible in four independent trials. Nuclear morphology of splenocytes was examined by staining with Hoechst dye 33342 24 h after irradiation. Thymocytes were inoculated at 4×10^6 cells ml^{-1} , exposed to γ -irradiation (10 Gy) and their survival determined as above.

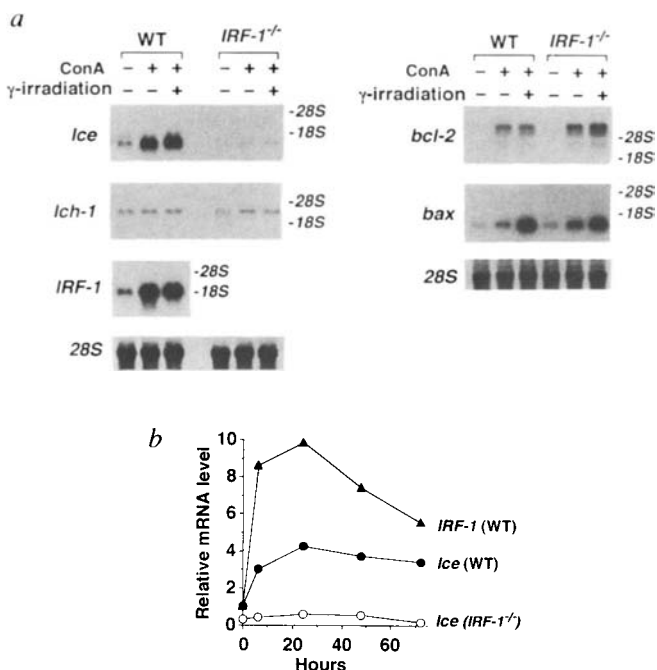
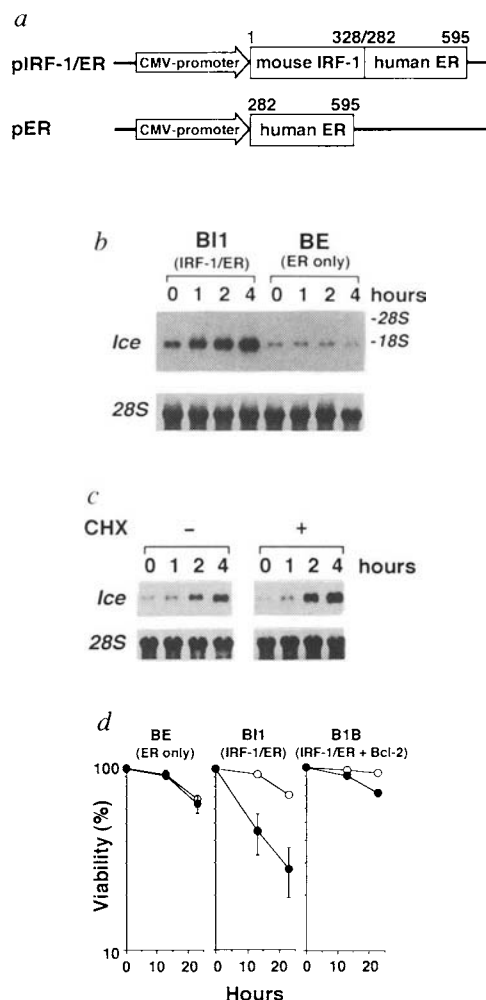


FIG. 2 Expression of apoptosis-related genes and *IRF-1* in splenocytes. **a**, RNA blotting analysis of RNA isolated from wild-type and *IRF-1*^{-/-} splenocytes without stimulation, after conA stimulation, and after conA stimulation followed by γ -irradiation (10 Gy). 28S ribosomal RNA shows that equivalent amounts of total RNA were analysed. **b**, Expression of *Ice* and *IRF-1* mRNAs following stimulation of wild-type (WT) or *IRF-1*^{-/-} splenocytes by conA. Levels of mRNA are relative to those observed in non-stimulated wild-type cells.

METHODS. Cells were cultured and treated as described above. Total RNA (5 μg) was prepared by guanidinium thiocyanate/CsCl-centrifugation and subjected to RNA blotting analysis as described¹¹. 28S rRNA was visualized by staining filter with methylene blue. Probes were generated by random-primer labelling of complementary DNAs for each of the genes shown. mRNA levels were quantified using a Fujix BAS 2000 image analyser.

lymphocytes⁸. Expression increased four- to fivefold after 3 days and was not further augmented by γ -irradiation. Furthermore, the timing of *Ice* gene induction following stimulation with conA was correlated closely with that of *IRF-1* (Fig. 2b). In contrast, in *IRF-1*^{-/-} splenocytes both constitutive and induced expression of *Ice* mRNA was severely impaired (Fig. 2a, b). The difference in mRNA expression levels between the wild-type and *IRF-1*^{-/-} splenocytes after stimulation with conA reached a maximum of 20-fold (Fig. 2b). In contrast to splenocytes, expression of *Ice* mRNA in both wild-type and *IRF-1*^{-/-} thymocytes was too low to quantify precisely (data not shown).

To further examine the possibility that IRF-1 is a transactivator of *Ice*, we constructed an expression vector encoding an oestrogen-regulatable derivative of IRF-1²⁰⁻²² (Fig. 3a). This (pIRF-1/ER) and a control construct (pER) were transfected into the cell line BER2 (ref. 23), a derivative of the IL-3-dependent murine pro-B cell line BAF/B03, and stable clones were isolated. As shown in Fig. 3b, treatment with β -oestradiol resulted in the induction of *Ice* mRNA in B11 cells, transfected with pIRF-1/ER, but not in the control BE cells, transfected with pER. β -oestradiol-induced *Ice* gene expression was also observed in several other clones expressing IRF-1/ER (data not shown), and occurred in the presence of cycloheximide (Fig. 3c). The latter result supports the idea that transactivation by IRF-1 is direct. Significantly, activation of IRF-1/ER in this system increased the susceptibility of these cells to radiation-induced apoptosis (Fig. 3d). Furthermore, the apoptotic response to irradiation in this IRF-1-inducible system was inhibited by the concomitant expression of the *bcl-2* gene (B1B cells; Fig. 3d), as had been observed previously in mature T lymphocytes⁴.



We found that a luciferase reporter plasmid driven by the mouse *Ice* promoter could be induced four- to fivefold when cotransfected with *IRF-1* in a transient assay (data not shown). The region of the *Ice* promoter most highly conserved between mouse and human is a 14-bp sequence, CTTTCAGTTTCAGT, located -41 to -28 nucleotides from the initiator methionine codon^{24,25}. This sequence conforms completely to a consensus IRF-E¹², is bound specifically by *in vitro*-translated IRF-1 in a gel mobility shift assay, and is sufficient to confer efficient IRF-1 inducibility when inserted into the promoter region of a luciferase reporter plasmid (data not shown).

Our results clearly delineate two pathways governing DNA damage-induced apoptosis in lymphocytes of the T lineage. One pathway functions in thymocytes and requires p53, whereas the other functions in mitogen-stimulated mature T lymphocytes and requires IRF-1. The utilization of these distinct pathways could be due to differences either in the maturation stage or in the proliferative state of these two types of lymphocytes⁴. Interestingly, IRF-1 and p53 also regulate expression of different apoptosis-related genes; *Ice* is induced by IRF-1, whereas *bax* is induced, and *bcl-2* is repressed, by p53 (ref. 26). Recent studies have shown that *Ice* is not required for γ -irradiation-induced apoptosis in thymocytes²⁷. In this regard, it would be interesting to examine DNA damage-induced apoptosis in activated mature T lymphocytes that lack a functional *Ice* gene. Overexpression of *Ice* can induce apoptosis¹⁵, but we have found that IRF-1-dependent upregulation of *Ice* is, by itself, insufficient to trigger apoptosis. There appears to be a signal(s) induced by DNA damage that is required in addition to IRF-1, and which could conceivably function by activating the latent ICE, for example

FIG. 3 Activation of ectopically expressed IRF-1 in BER2 cells causes induction of *Ice* gene and enhances susceptibility to γ -irradiation. **a**, Vectors encoding the oestrogen-regulatable IRF-1 chimera (pIRF-1/ER) and control (pER). Numbers represent amino acids encoded by each cDNA fragment. **b**, RNA blotting analysis of *Ice* mRNA collected from cells expressing IRF-1/ER (B11 cells) or ER (BE cells) at various times following oestrogen treatment. **c**, *Ice* mRNA levels in B11 cells following treatment with oestrogen and with or without cycloheximide (CHX). **d**, Cell viability at times following γ -irradiation of BE, B11 and B1B cells pretreated (filled circles) or not treated (open circles) with oestrogen. Vertical bars represent standard deviation from three independent trials.

METHODS. A chimaeric cDNA joining the mouse IRF-1 (ref. 5) and the hormone-binding domain of the human oestrogen receptor²⁰ and a cDNA encoding the hormone-binding domain alone were inserted into the expression vector CDM8 to generate the vectors pIRF-1/ER and pER, respectively. These vectors were transfected into BER2 cells²³ along with a hygromycin-resistance gene, and the stable cell lines B11 (expressing IRF-1/ER) and BE (expressing ER) were selected. Cells were treated by the addition of 1 μ M β -oestradiol to the culture media. In **c**, cycloheximide (100 μ g ml⁻¹) was added 10 min before the treatment with β -oestradiol. RNA was collected at the times indicated and 5 μ g was subjected to blotting analysis. For determination of susceptibility to γ -irradiation, cells were treated (filled circles) or not treated (open circles) with 1 μ M β -oestradiol and then subjected to γ -irradiation (10 Gy) 6 h later. B1B cells were generated by transfection of B11 cells with a human *bcl-2* expression vector (pSVBT)³⁰ along with a puromycin-resistance gene. Cell viability was determined by trypan blue exclusion at times following irradiation and calculated as percentage viable versus total cell number. DNA fragmentation assay indicated that loss of viability was due to apoptosis (data not shown).

by proteolysis⁹. However, genes other than *Ice* may also mediate the effects of IRF-1 on apoptosis.

The possible involvement of IRF-1 as a tumour suppressor had previously been reported in the context of the development of myelodysplastic syndrome (MDS) and leukaemia^{13,14}. In view of the results reported here, it may be interesting to investigate the susceptibility of *IRF-1*^{-/-} mice to radiation-induced tumours, as well as the status of the *IRF-1* gene in other, particularly lymphocyte-derived, forms of human neoplasms. □

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Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction

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A serine/threonine kinase, named protein kinase B (PKB)¹ for its sequence homology to both protein kinase A and C, has previously been isolated. PKB, which is identical to the kinase Rac², was later found to be the cellular homologue of the transforming v-Akt³. Here we show that PKB is activated by stimuli such as insulin, platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Activation of PKB was inhibited by the phosphatidylinositol-3-OH kinase (PI(3)K) inhibitor wortmannin and by coexpression of a dominant-negative mutant of PI(3)K. PDGF receptor mutants that lack detectable associated PI(3)K activity also fail to induce PKB activation. PKB kinase activity is correlated with phosphorylation of PKB on serine. Finally, we show that a constructed Gag-PKB fusion protein, homologous to the *v-akt* oncogene, displays significantly increased ligand-independent kinase activity. Furthermore, this activity is sufficient to activate the p70 S6-kinase (p70^{S6k}). These results suggest a role for PKB in PI(3)K-mediated signal transduction.

Rat-1 fibroblasts or insulin receptor overexpressing NIH3T3 cells (A14), transiently expressing an epitope-tagged version of PKB (HA-PKB), were treated with different stimuli, and HA-PKB was isolated. Increased HA-PKB kinase activity was observed after treatment with PDGF, EGF, insulin and bFGF (Fig. 1a). Activation of a G_i-protein-coupled receptor by lysophosphatidic acid (LPA) or activation of PKC by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) did not enhance HA-PKB activity in these cells. Activation of HA-PKB by PDGF occurred at a half-maximal dose of 2 ng ml⁻¹ and was rapid (maximal activation within 2–5 min) but transient (return to basal within 30–40 min).

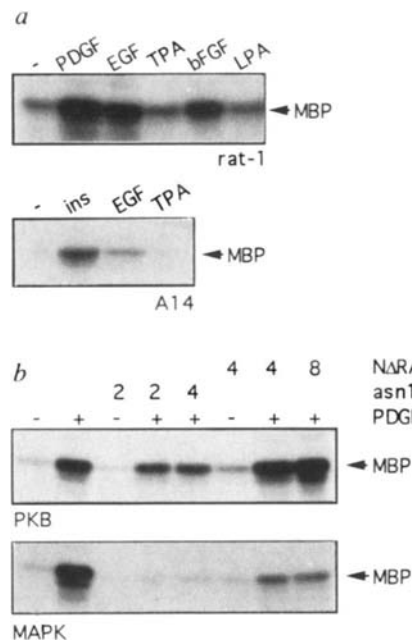
We analysed the involvement of the p21^{ras} (Ras) signalling pathway in PDGF-induced PKB activation. HA-PKB was coex-

pressed with either the Ras dominant-negative mutant Ras^{asn17} or the *raf-1* dominant-negative mutant NΔraf. Expression of Ras^{asn17} and NΔraf inhibited activation of HA-MAPK, as expected^{4,6} (Fig. 1b), but little or no inhibition of PDGF-induced HA-PKB activation was observed. Also Ras^{asn17} expression did not inhibit insulin- and EGF-induced HA-PKB activity in A14 cells (not shown). Activation of Ras and/or Raf-1 is apparently not essential to PDGF-induced PKB activation.

We then analysed whether PKB activation might be part of a PI(3)K-dependent signalling pathway. First, we tested the effect of the PI(3)K inhibitor wortmannin⁷ on HA-PKB activation (Fig. 2a). Pretreatment with wortmannin inhibited HA-PKB activation by PDGF, with only a small effect on HA-MAPK activation at higher doses of wortmannin. Both the 50% inhibitory concentration (IC₅₀) for PKB inhibition and PI(3)K activity present in antiphosphotyrosine immunoprecipitates was approximately 10 nM (not shown). Second, we analysed NMuMG cells expressing the PDGF receptor (NmR1), cells expressing a kinase-insert domain deletion mutant (ΔKi), no longer binding NCK, Grb2, p120GAP and PI(3)K, and cells expressing a mutant defective in p120GAP binding (5W)⁸. Treatment of cells with PDGF or EGF results in Ras activation and activation of MAP kinase (see refs 6, 9; and Fig. 2b). PDGF failed to activate HA-PKB in the ΔKi mutant (Fig. 2b), owing to the mutant PDGF receptor expressed, because EGF activated HA-PKB normally in this cell line (Fig. 2b). To investigate in more detail the possible involvement of PI(3)K in PKB activation we further analysed PKB activation in PAE cells expressing different PDGF receptor point mutants. These included the single PDGF receptor point mutations Y740F and Y751F, as well as the double-mutant Y740F/Y751F¹⁰. These mutants show reduced ability to stimulate PI(3)K activity. The different cell lines were transfected, and PDGF-induced PI(3)K and HA-PKB activity were compared (Fig. 2b). From this we conclude that activation of HA-PKB fully correlates with the ability of these receptors to activate PI(3)K. Finally, we investigated whether HA-PKB activation could be blocked by coexpression of a deletion mutant of the p85 subunit of PI(3)K (Δp85), which was previously shown to inhibit receptor-induced PI(3)K activity¹¹. Coexpression of Δp85 inhibited PDGF-induced HA-PKB activation, but had no effect on PDGF-induced HA-MAPK activation (Fig. 2c). Taken together, these data demonstrate that PI(3)K activation is essential for PKB activation.

To study the mechanism of PKB activation we analysed whether PKB was regulated by phosphorylation. HA-PKB

FIG. 1 PKB is activated by receptor tyrosine kinases, and activation is not inhibited by Ras^{asn17} or NΔraf coexpression. **a**, HA-PKB activity in transfected Rat-1 cells following stimulation with different growth stimuli. HA-PKB activity was analysed from extracts of untreated Rat-1 or A14 cells (NIH3T3 overexpressing the human insulin receptor), and Rat-1 or A14 cells treated with the indicated stimuli (20 ng ml⁻¹ PDGF; 20 ng ml⁻¹ EGF; 100 nM TPA; 20 ng ml⁻¹ bFGF; 1 μM LPA; 100 nM insulin (ins)). Before stimulation, cell cultures were cotransfected with HA-PKB (2 μg per dish) in the presence of 3 μg of the vector plasmid. All indicated stimuli activate MAP kinase in these cells⁵, so lack of activation is not due to a lack of responsiveness of Rat-1 or A14 cells to these stimuli. **b**, HA-PKB activity or HA-MAP-kinase activity was analysed from extracts of untreated Rat-1 cells or PDGF (20 ng ml⁻¹) treated Rat-1 cells. Before treatment Rat-1 cells were cotransfected with HA-PKB (2 μg) in the presence of Ras^{asn17} or NΔraf. **METHODS**. A full-length protein kinase B cDNA was isolated from a bovine cDNA library as previously described¹. A 2.4-kb EcoRI fragment containing the complete PKB cDNA was cloned into the mammalian expression vector pSG5 (ref. 17) generating the vector pSG5-PKB. PKB was N-terminally tagged with a 9-amino-acid peptide (HA1) from influenza virus haemagglutinin¹⁸ generating the plasmid pSG5-PKB^{TAG}. The N-terminal tag is recognized by the monoclonal antibody 12CA5. The use of HA-MAP kinase, Ras^{asn17} and NΔraf have been described previously⁶. Approximately 2 × 10⁵ Rat-1 cells were seeded per 5 cm dish in DMEM supplemented with 10% FCS and transfected using the Ca(PO₄)₂ technique. For PKB activity determination, cells were washed twice in ice-cold PBS and HA-PKB was isolated as previously described for HA-MAP kinase⁶. After the final wash the precipitate was incubated in 25 μl kinase buffer: 50 mM Tris, pH 7.5; 10 mM MgCl₂; 1 mM DTT; 1 μM PKI; 50 μM rATP; 10 μg myelin basic protein (MBP) and 3 μCi ³²P-γATP. Kinase reaction was for 25 min at room temperature with continuous shaking. Reaction was stopped by the addition of 7 μl of



5× Laemmli sample buffer. The reaction was separated by electrophoresis on 15% SDS-PAGE. MBP phosphorylation was detected by autoradiography. Determination of HA-MAP kinase activity has been described previously⁶.

immunoprecipitated from transfected, ³²P-orthophosphate-labelled cells appeared in two phosphorylated forms. Phosphorylation of the faster migrating form was constitutive and not induced by PDGF, but phosphorylation of the slower migrating form of PKB was induced by PDGF and inhibited by pretreatment with wortmannin (Fig. 3a). Phosphoamino-acid analysis showed that phosphorylation was mainly on serine (Fig. 3b). This phosphorylation appeared essential for PKB kinase

activity, because treatment of active PKB immune complexes with phosphatase abolished the ability of PKB to phosphorylate substrate. Furthermore, after phosphatase treatment all PKB was present as the faster migrating form (Fig. 3c).

As with PKB, activation of p70^{S6k} by PDGF is inhibited by wortmannin and the Δp85 mutant¹² (Fig. 2c). In addition, the various receptor mutants (ΔKi; Y740F/Y751F and Y751F) demonstrate reduced activation of PI(3)K, PKB and p70^{S6k}. In

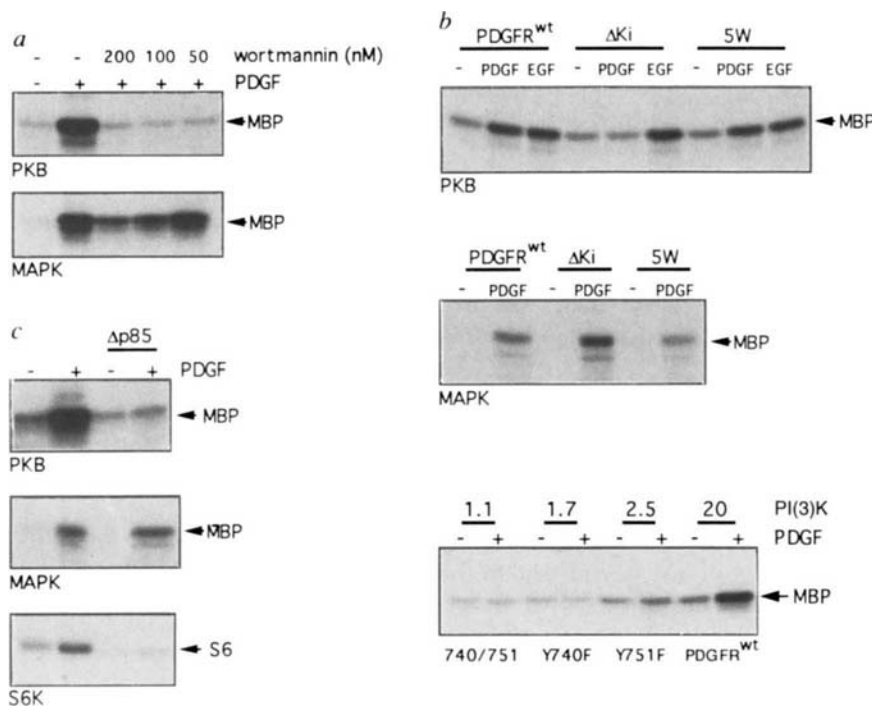


FIG. 2 PI(3)K activity is essential for PKB activation. **a**, Inhibition of PDGF-induced HA-PKB activation by wortmannin. HA-PKB activity was analysed from extracts of transfected Rat-1 cells pretreated with the indicated concentration of wortmannin and subsequently stimulated with PDGF. **b**, HA-PKB and HA-MAP kinase activity was measured in transfected NMuMG or PAE cells expressing different PDGF-receptor mutants. Cells were stimulated before extraction with the indicated growth factors. In the case of the PAE cells expressing the various PDGF receptor mutants, PI(3)K activity present in antiphosphotyrosine immunoprecipitates was measured in parallel and is expressed as fold induction (third panel). **c**, Inhibition of PDGF-induced HA-PKB and HA-p70^{S6k}, but not HA-MAP kinase, activation by Δp85. The PDGF-induced activities of HA-PKB, HA-MAPK and HA-p70^{S6k} were measured in the presence or absence of 8 μg Δp85 plasmid.

METHODS. Wortmannin pretreatment was for 10 min with the amounts indicated. PDGF treatment, cell lysis, immunoprecipitation and kinase assay were as Fig. 1. Culturing and characteristics of PDGF signalling of the various NMuMG and PAE cell lines have been described previously^{6,9,10}. Determination of S6kinase activity has been described previously⁶. Phosphorylated S6 protein was separated by electrophoresis on 12.5% SDS-PAGE and detected by autoradiography.

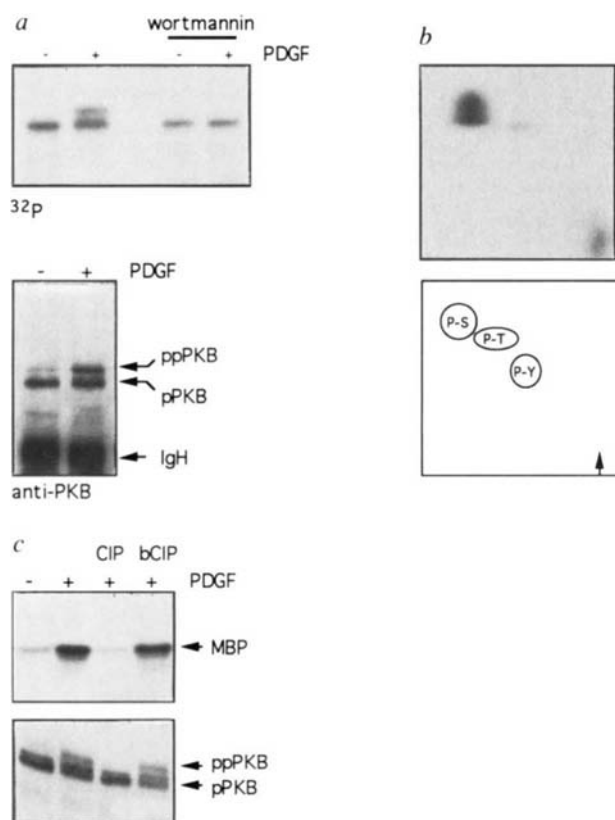
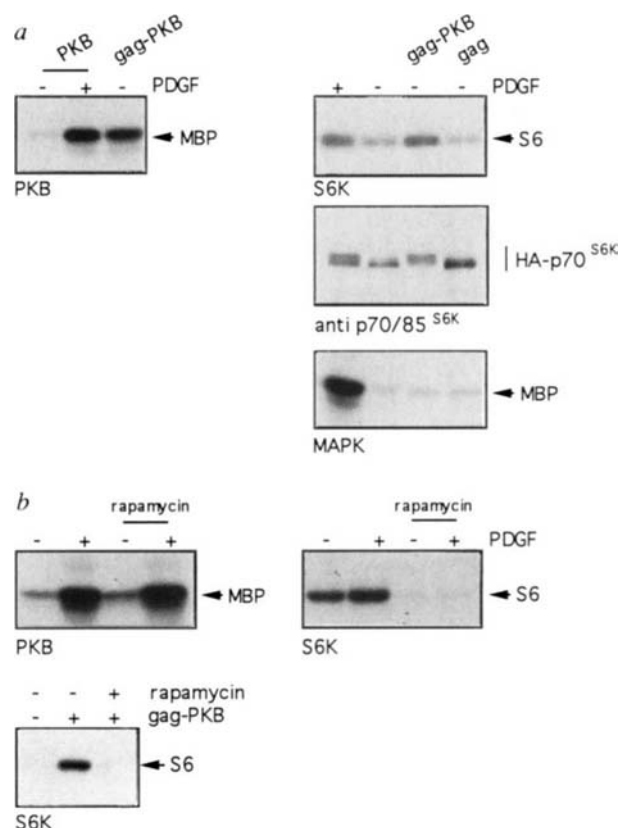


FIG. 3 *In vivo* phosphorylation of HA-PKB. **a**, PDGF-induced phosphorylation of PKB and the effect of wortmannin were analysed by immunoprecipitation of *in vivo* 32 P-labelled HA-PKB from transfected Rat-1 cells treated with the indicated stimuli. After separation of proteins by polyacrylamide gel electrophoresis and blotting of proteins to polyvinylidene difluoride (PVDF) membrane, labelled HA-PKB was visualized by autoradiography (top). The position of HA-PKB was determined by immunoblotting (bottom). **b**, The phospho-amino acid (PAA) content of PKB was determined in parallel experiments in which 32 P-labelled HA-PKB was excised from the PVDF membrane followed by acid hydrolysis and two-dimensional chromatography. The position of the phosphoserine, phosphothreonine and phosphotyrosine standards are indicated. The autoradiograph shown is the PAA analysis of the upper phosphorylated band from PDGF stimulated cells. PAA analysis results of all other bands were essentially identical to the one shown here. **c**, The effect of treatment with calf intestinal phosphatase (CIP) on HA-PKB activity and gel-shift was measured by incubating immunoprecipitates of active HA-PKB with either CIP or heat-inactivated CIP (bCIP), followed by an *in vitro* kinase assay or analysis on SDS-PAGE.

METHODS. Transfected Rat-1 cells were labelled overnight with 1 mCi 32 P-orthophosphate per ml medium. Following stimulation with PDGF (20 ng ml $^{-1}$) for 7 min either with or without wortmannin (50 nM) pretreatment (10 min), cells were lysed and HA-PKB was precipitated as in Fig. 1. After separation of proteins by SDS-PAGE on 8% polyacrylamide gels, proteins were transferred to PVDF membranes. The position of labelled proteins was first determined by autoradiography and in parallel by incubation of the transferred protein with polyclonal anti-PKB serum and detection with HRP-conjugated secondary antibody and enhanced chemiluminescence (Amersham). PAA analysis was performed according to standard procedures¹⁹. In **c**, active HA-PKB was immunoprecipitated from PDGF-treated HA-PKB transfected Rat-1 cells. Precipitates were incubated with either 5 units of CIP for 5 min at 37 °C or the same amount of heat-inactivated CIP. After CIP incubation, samples were washed extensively and assayed for kinase activity as in Fig. 1. Parallel samples were analysed by gel electrophoresis.

FIG. 4 PKB activates p70^{S6k} at a site upstream of rapamycin inhibition. **a**, Gag-PKB activity was analysed from extracts of untreated Rat-1 cells and compared with unstimulated and PDGF-stimulated HA-PKB activity. Expression of Gag-PKB and PKB was similar as determined by western blotting of total extracts with anti-PKB serum. Comparison of total protein extracts and immunoprecipitated protein showed that HA-PKB and Gag-PKB were precipitated with equal efficiency (not shown). HA-p70^{S6k} and HA-MAP kinase activity were analysed from extracts of untreated Rat-1 cells or cells treated with PDGF. This was compared with activity present in extracts of Rat-1 cells that before analysis were cotransfected with either pSG5-GagPKB or pSG5-Gag as a control. **b**, The effect of the immunosuppressant rapamycin on HA-p70^{S6k} activity and HA-PKB was compared by measuring the activity of untreated, PDGF-treated or Gag-PKB cotransfected cells either with or without pretreatment with rapamycin.

METHODS. The plasmid pSG5-PKBGag was constructed by ligating an 800-bp MoMuLV cDNA fragment encoding the p30 capsid protein³ in frame with the initiation codon of PKB utilizing an engineered NcoI site. This was subsequently cloned into pSG5 and, upon transfection of mammalian cells, generates a fusion protein of $M_r \sim 85K$ as detected by western blotting of total protein extracts with both anti-PKB and anti-Gag antibodies (not shown). Activity measurements using MBP as a substrate were performed on immunoprecipitated protein as in Fig. 1. The Gag-PKB fusion protein was immunoprecipitated using a MoMuLV-Gag-specific antiserum. In **a**, HA-p70^{S6k} or HA-MAP kinase were cotransfected with 3 μ g of either pSG5, pSG5-GagPKB or pSG5-Gag plasmid. In **b**, Rat-1 cells transfected with HA-PKB or HA-p70^{S6k} were pretreated with 25 ng ml $^{-1}$ rapamycin for 10 min followed by PDGF (20 ng ml $^{-1}$) treatment for 10 min. In the case of Gag-PKB cotransfection, rapamycin (25 ng ml $^{-1}$) treatment was also for 10 min.



contrast, the Y740F mutant, which also displays reduced PI(3)K and PKB activation, activates p70^{S6k} normally, indicating that, in this cell-type, Y740F activates a PI(3)K-independent pathway to activate p70^{S6k} (ref. 12). To investigate whether PKB mediates PI(3)K-dependent p70^{S6k} activation, we constructed a Gag-PKB fusion protein, similar to the *v-akt* oncogene, as a putative constitutively active form of PKB. Indeed, Gag-PKB displayed a much higher basal level of kinase activity than HA-PKB (Fig. 4a). This increased level could still be induced by PDGF and was sensitive to wortmannin, indicating that Gag-PKB is still under the control of upstream regulatory signals (data not shown). Most importantly, in co-transfection assays the elevated levels of PKB activity resulted in an increase of p70^{S6k} activity, as well as an increase in p70^{S6k} phosphorylation (Fig. 4a). In contrast, Gag-PKB had no effect on the activation of MAP kinase, arguing against an explanation that Gag-PKB activates p70^{S6k} indirectly by production of autocrine factors. Further proof for PKB being a mediator would be inhibition of p70^{S6k} activation by a dominant-negative mutant of PKB. We have tried a kinase-dead mutant of PKB and, although we did observe inhibition in some experiments, the results were not conclusive. In this respect this mutant did not differ from most other kinase-dead versions of kinases, which generally act as very poor dominant-negatives. Irrespective of this, the observations that both PKB and p70^{S6k} are downstream elements of PI(3)K, and that Gag-PKB activates p70^{S6k}, strongly suggests that p70^{S6k} may be a downstream element of PKB-mediated signalling.

Growth factor-induced p70^{S6k} is also inhibited by the macrocyclic rapamycin at a site downstream from PI(3)K¹². We analysed the effect of rapamycin on activation of p70^{S6k} induced by PDGF and Gag-PKB. Treatment of the transfected cells with rapamycin clearly ablated p70^{S6k} activation by both PDGF and Gag-PKB, but had no effect on PKB activation itself (Fig. 4b). This indicates that PKB is situated upstream of, or parallel to, the site of rapamycin inhibition.

After submission of this manuscript, Franke *et al.* also reported PDGF-induced activation of the *Akt* proto-oncogene¹³. In agreement with our results they demonstrate a role for PI(3)K in *c-akt*/PKB activation. However, here we report that PKB activation is not restricted to PDGF alone but is induced by a variety of PI(3)K-activating stimuli. With respect to the role of Ras in PKB activation, it appears clear from both sets of data that Ras activation is not sufficient. There is a discrepancy as to whether Ras is necessary for PKB activation. This may be due to differences in cell type, and might reflect the close interplay between PI(3)K and Ras, as recent studies have positioned PI(3)K both upstream¹⁴ and downstream¹⁵ of Ras.

In conclusion, we have shown that growth factor-induced PKB activation is mediated by PI(3)K. Furthermore, we obtained evidence that one of the responses of PKB activation is the activation of p70^{S6k}. These results not only identify PKB as an element of a growth factor-induced signal transduction pathway, but also imply that the PI(3)K pathway is sensitive to oncogenic conversion. Recently, one other element downstream from PI(3)K, the small GTPase Rac, has been shown to induce oncogenic transformation when transfected in its active form¹⁶. Together these results show how important the PI(3)K pathway may be in the development of human cancer. □

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Recognition of bZIP proteins by the human T-cell leukaemia virus transactivator Tax

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HUMAN T-cell leukaemia virus type I (HTLV-I) Tax protein increases the DNA binding of many cellular transcription factors that contain a basic region-leucine zipper (bZIP) DNA-binding domain^{1–3}. bZIP domains comprise a leucine-rich dimerization motif and a basic region that mediates DNA contact. How Tax recognizes diverse bZIPs is not understood. Here we show that no specific sequence of the leucine zipper is required for a Tax response. In contrast, the basic region is essential for the Tax-mediated DNA-binding increase, which can be eliminated by single substitutions of several conserved amino acids. Surprisingly, Tax alters the relative affinity of a bZIP for different DNA binding sites. Thus, through recognition of the conserved basic region, Tax increases DNA binding and modifies DNA site selection. Tax provides a model for how a single auxiliary factor can regulate multiple sequence-specific DNA-binding proteins.

To delineate the element(s) of the bZIP domain required for Tax function, we analysed the DNA-binding activity of a series of bZIP derivatives by using a mobility-shift assay. The magnitude of the Tax response is influenced by bZIP concentration¹, and therefore the experiments described below were done over a range of bZIP concentrations, at least two of which are shown. Although a Tax-bZIP-DNA complex forms in solution, Tax dissociates under standard native gel electrophoresis conditions^{1, 3}.

We began by examining the role of the leucine zipper. The synthetic peptide BR-CC contains the basic region of the yeast GCN4 bZIP protein attached to an idealized coiled-coil dimerization motif⁴. BR-CC contains the canonical leucine repeat, but otherwise the dimerization domains of GCN4 and BR-CC are dissimilar (Fig. 1a, bottom). BR-CC dimerizes and binds an AP-1 site with an affinity similar to GCN4 (ref. 4). Figure 1a shows that Tax stimulated the DNA binding of BR-CC and GCN4 to comparable levels.

We next examined whether the leucines were important for Tax recognition. The ZTA protein of Epstein-Barr virus is a highly divergent member of the bZIP family^{5, 6}. The ZTA dimerization domain diverges from a typical leucine zipper, yet assumes a coiled-coil structure⁷ that supports dimerization and DNA binding. Figure 1b shows that Tax efficiently stimulated the binding of a GST ZTA derivative (see Fig. 1 legend) to the

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collagenase promoter AP-1 site (lanes 1–4). Similarly, Tax increased the DNA binding of a hybrid protein containing the human Fos basic region fused to the ZTA dimerization domain⁷ (lanes 5–8). The combined data of Fig. 1 indicate that no sequence of the leucine zipper is required for Tax function.

The fact that Tax does not recognize the leucine zipper raised the possibility that this domain was dispensable for Tax-responsiveness. The synthetic peptide br1ss⁸ contains two GCN4 basic regions joined by a disulphide linkage. Figure 1c (lanes 1–4) shows that Tax did not stimulate the DNA binding of br1ss under conditions in which the DNA binding of GCN4 was markedly enhanced (lanes 5–10). Thus Tax function requires a protein

with an appropriate dimerization domain, consistent with the ability of Tax to enhance dimerization¹.

To test whether the conserved basic region is the target for Tax, we first analysed a chimaeric protein, λ -ZIP, in which the helix–turn–helix DNA binding domain of bacteriophage λ repressor cI was fused in-frame to the GCN4 leucine zipper⁹. λ -ZIP efficiently dimerizes through the leucine zipper and binds to the O_R1 site of the bacteriophage λ P_{RM}/P_R promoter. Figure 2a shows that Tax failed to stimulate the DNA binding of λ -ZIP, indicating that the leucine zipper is not sufficient for Tax-responsiveness and suggesting an essential role for the basic region. To confirm this, we analysed GCN4 mutants with single

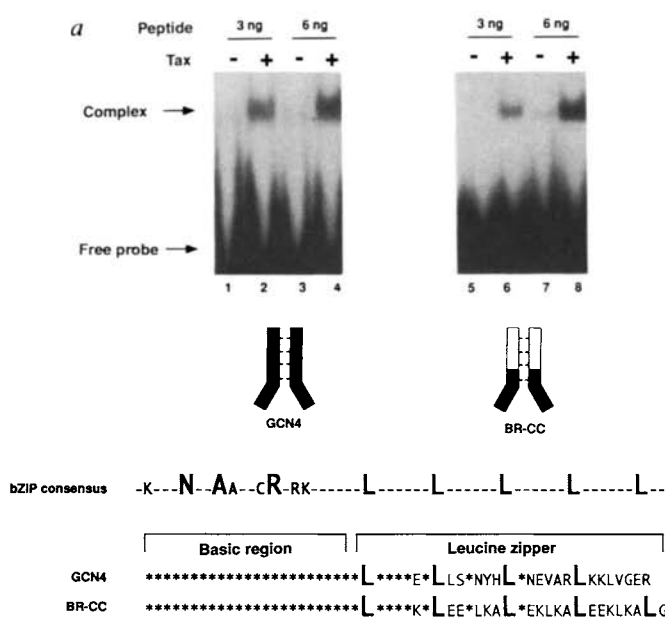
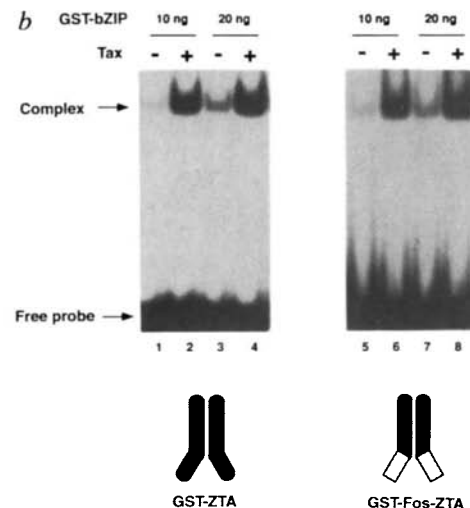
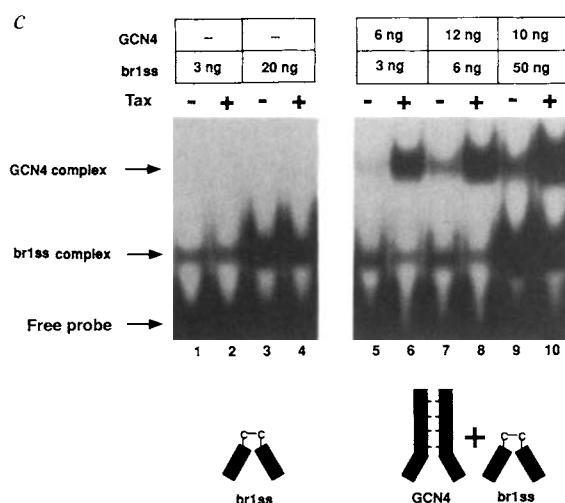


FIG. 1. Role of the leucine zipper in Tax responsiveness. **a**, BR-CC peptide. GCN4-bZIP (ref. 8) and BR-CC (ref. 4) peptides were analysed for DNA binding in the absence (–) or presence (+) of 200 ng H6-Tax. The ³²P-DNA probe contained the collagenase AP-1 site (5'-AGCTTGGTGACTCATCCGG-3'). The positions of the free probe and DNA–protein complexes are indicated. Representations of GCN4 bZIP and BR-CC are shown below the autoradiogram: black, GCN4 regions; white, BR-CC idealized coiled-coil. In the bZIP consensus sequence: dashes, non-conserved amino acids; small letters, amino acids conserved in the majority of the bZIPs' large letters, amino acids conserved in almost all bZIPs. In the GCN4 and BR-CC sequences: asterisks, residues that are identical between GCN4 and BR-CC; bold, leucines in the dimerization domain; small letters, residues that differ between GCN4 and BR-CC. **b**, ZTA and Fos-ZTA. Schematic representations of the ZTA and Fos-ZTA bZIP domains are shown: black, ZTA bZIP. Fos-ZTA bZIP comprises the ZTA dimerization domain (black) and the Fos basic region (stippled). Amino-acid sequences of bZIP domains of Fos, ZTA and Fos-ZTA are shown below. Positions of the leucines and amino acids replacing leucines are in bold. **c**, br1ss peptide. Tax-responsiveness of br1ss dimers was tested either alone (lanes 1–4) or in the presence of GCN4 (lanes 5–10). Amino-acid sequences of GCN4-bZIP and br1ss peptides are shown below.

METHODS. H6-Tax protein was expressed and purified as described¹⁶. GCN4-bZIP and BR-CC binding reaction mixtures contained in 1 μ g poly(dI):poly(dC), 1 μ g BSA, 0.5 $\times$ D buffer (50 mM KCl, 100 ml l⁻¹ glycerol, 0.05 mM EDTA) and 0.1 ng of ³²P-labelled DNA probe. After incubation at room temperature for 15 min, DNA–protein complexes were resolved on a 5% polyacrylamide gel, 0.5 $\times$ TBE. pYNC76 and pYNC129 containing zta complementary DNA and c-fos-zta hybrid, respectively⁷, were used to amplify the minimal bZIP domain of ZTA (residues 175–229) and Fos-ZTA (residues 133–162 (Fos)/197–229 (ZTA)) by polymerase chain



	Basic region	Leucine zipper
Fos	RRERNKMAAAKCRNRRRLTDTLQAETDQLEDEKSA	LQTEIANLLEKEKELE
ZTA	KRYKNRVASRKCRAKFKQLLQH	YREVAALKSSENDRLRLLLKQMCPSLVD
Fos-ZTA	RRERNKMAAAKCRNRRRLTDTLQAETDQLEDEKSA	LQTEIANLLEKEKELE



	Basic region	Leucine zipper
GCN4	PESSDPAALKRARNTAARRSRARKLQRMKQLEDKVEELLKSNYHLENEVARLKKLVGER	
GCN4-br1ss	PESSDPAALKRARNTAARRSRARKLQRMKQggc	

reaction (PCR). PCR products were cloned in the pGEX-2T vector for expression as GST derivatives. Dimers of br1ss were generated as described⁸. Binding of br1ss was analysed as above except that incubation was performed at 4 $^{\circ}$ C. DNA–protein complexes were resolved on an 8% polyacrylamide gel at 4 $^{\circ}$ C.

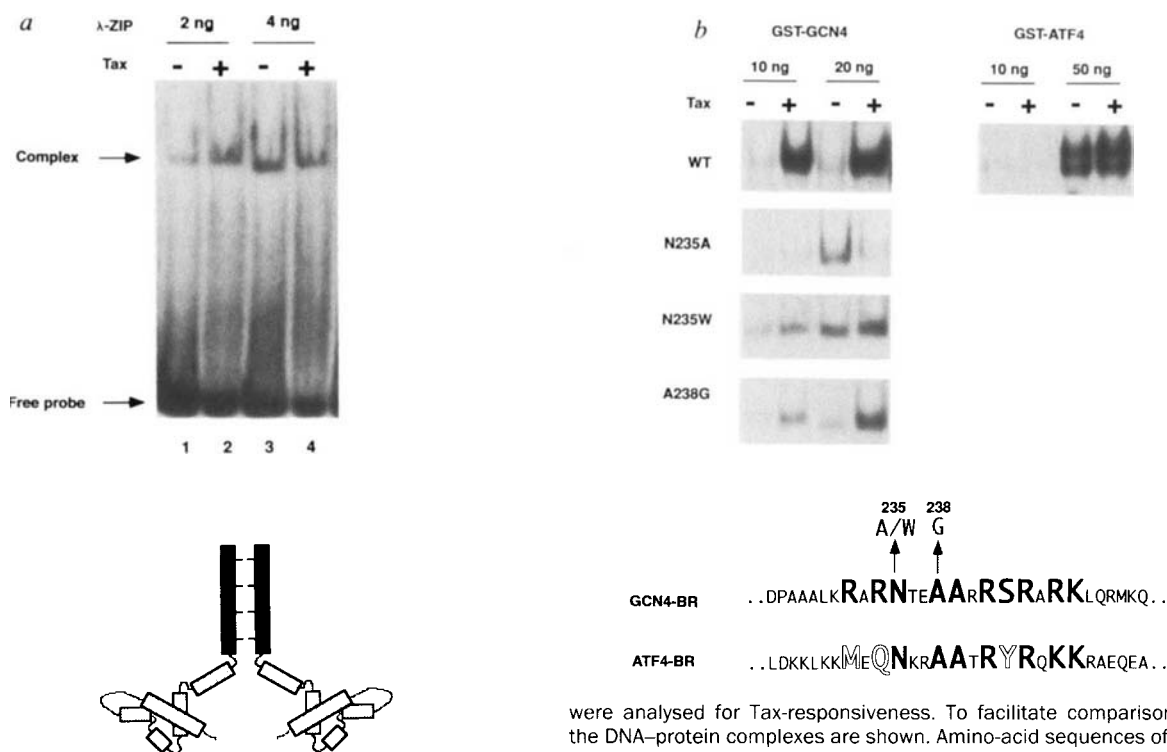


FIG. 2 Role of the basic region in Tax responsiveness. *a*, Tax requires the bZIP basic region. The λ-ZIP chimaeric protein comprising the λ repressor DNA-binding domain (residues 1–101) fused to the GCN4 leucine zipper (residues 250–281) was tested in the absence (–) or presence (+) of Tax. A schematic representation of λ-ZIP is shown below: black, GCN4 leucine zipper; light grey: λ repressor helix–turn–helix DNA-binding domain. *b*, Mutations in the basic region abolish Tax responsiveness. Three different GCN4 mutants and an ATF4 derivative

were analysed for Tax-responsiveness. To facilitate comparison, only the DNA–protein complexes are shown. Amino-acid sequences of GCN4 and ATF4 basic regions are shown below. Amino acids conserved among bZIPs are in bold. Positions of GCN4 mutants are indicated. ATF4 amino acids that diverge from the consensus are in outline. METHODS. The λ-ZIP protein was expressed as described⁹ and tested for DNA binding to a ³²P-labelled DNA probe containing the O_R1 site of P_{RM}/P_R promoter (5′-TATCACC GCCAGAGTA-3′). Plasmids carrying the wild type (WT) and GCN4 mutants^{10,11} were used to PCR-amplify the minimal bZIP region (residues 222–281). PCR products were cloned in the pGEX-2T vector for expression as GST derivatives. ATF4 (residues 1–351) was expressed as a GST fusion protein.

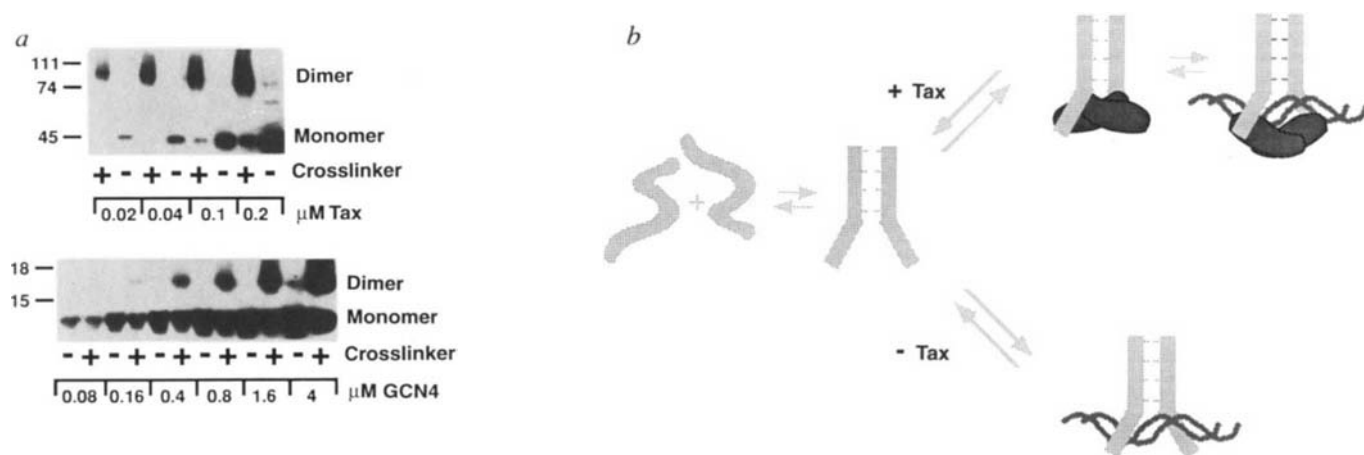


FIG. 3 Model for Tax-mediated stimulation of bZIP DNA binding. *a*, Glutaraldehyde protein crosslinking. The apparent dissociation constants for Tax (top) and GCN4 (bottom) dimers were estimated by glutaraldehyde protein crosslinking as described¹. Reaction mixtures containing various concentrations of Tax were treated with glutaraldehyde, the products fractionated by SDS–PAGE and detected by immunoblotting. The concentrations (μM) of the proteins are shown below the autoradiogram. The presence (+) or absence (–) of glutaraldehyde (crosslinker) is indicated. Size markers (relative molecular masses in thousands) are shown on the left. The monomer and dimer products are indicated on the right.

b, Model. By interacting with the bZIP basic region, a Tax dimer increases bZIP concentration. The increased concentration of bZIP dimer increases DNA binding. METHODS. Various concentrations of GCN4 peptide or H6-Tax were incubated at room temperature for 30 min in the absence or presence of glutaraldehyde (0.2 g l^{–1}). Products were resolved by 8% (Tax) and 15% (GCN4) SDS–PAGE, transferred onto a nitrocellulose membrane and detected with α-Tax (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) or αGCN4 polyclonal antibodies.

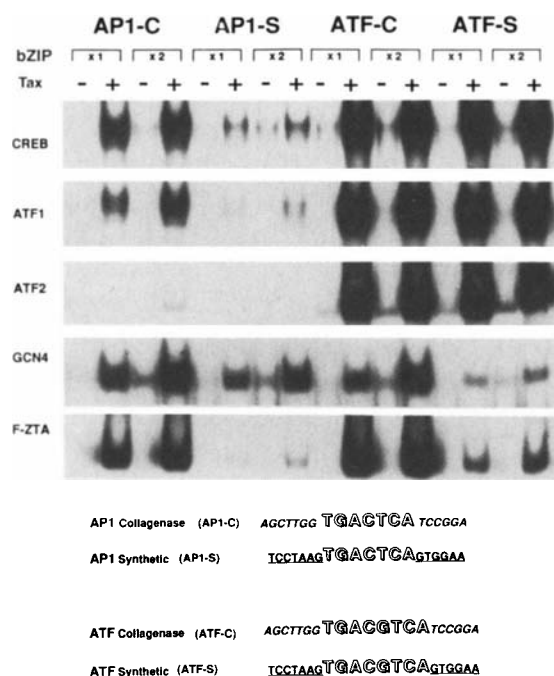


FIG. 4 Tax alters DNA-binding-site selectivity. GST derivatives of several bZIP proteins (CREB (residues 21–341), ATF1 (57–271), ATF2-bZIP, GCN4-bZIP, Fos-ZTA-bZIP) were tested for Tax responsiveness on four different DNA-binding sites. The relative concentration ($\times 1$ or $\times 2$) of a given bZIP in the reaction mixture is indicated. Sequences of AP1-C, AP1-S, ATF-C and ATF-S oligonucleotides are shown below. The AP1 and ATF core binding sites are shaded. AP1 collagenase flanking sequences are in *italics* and the synthetic polylinker sequences are underlined.

METHODS. GST derivatives were generated and purified as described¹. Gel shifts were performed as in Fig. 1.

substitutions in conserved residues of the basic region^{10,11}. Each of these single-substitution mutants bound to DNA with an affinity roughly comparable to that of the wild-type protein (Fig. 2b, and results not shown). However, DNA binding of GCN4 N235A, N235W and A238G mutants^{10,11} was not efficiently stimulated by Tax (Fig. 2b). Likewise, the human bZIP protein ATF4 (ref. 12), which possesses an atypical basic region that diverges at several residues from most bZIP proteins (Fig. 2b, bottom), was also not Tax-responsive (Fig. 2b).

Why does binding of Tax to the basic region promote bZIP dimerization? Figure 3a shows the use of previously described glutaraldehyde protein crosslinking assay to demonstrate that Tax is a very stable dimer with an apparent dissociation constant in the nanomolar range. By comparison, both protein crosslinking experiments (Fig. 3a, bottom; and ref. 1) and biophysical measurements^{13,14} indicate that the apparent dissociation constant of the GCN4 dimer is in the micromolar range. Thus we propose (Fig. 3b) that a Tax dimer interacts with the basic regions of a relatively unstable bZIP dimer, thereby stabilizing the dimeric form.

The fact that Tax interacts with the basic region, which mediates DNA contact, prompted us to investigate the DNA-binding specificity of Tax-bZIP complexes. We determined the relative affinity of several Tax-bZIP complexes for four different DNA-binding sites. The four DNA probes contained an AP-1 or an ATF consensus site flanked by sequences derived from either the collagenase promoter (AP1-C, ATF-C) or a synthetic polylinker (AP1-S, ATF-S) (Fig. 4, bottom). Consistent with previous results (see, for example, ref. 14), in the absence of Tax the relative affinity of a given bZIP protein for the four sites was comparable (within threefold; results not shown). In contrast, Fig. 4 shows that in the presence of Tax, the relative affinity of each bZIP for the four sites was dramatically different. For example, in the presence of Tax, both CREB and ATF1, which have identical bZIPs¹², bound efficiently to the AP1-C, ATF-C and ATF-S sites, but not to the AP1-S site. For ATF2, the Tax-mediated DNA binding increase occurred only with the ATF core site, regardless of flanking sequence (ATF-C, ATF-S). The combined results indicate that the Tax-mediated DNA binding increase depends upon the combination of the core-binding element and its flanking sequences. Remarkably, the optimal combination of core-binding site and flanking sequence differs for each bZIP tested.

In this study we have shown that the two bZIP subdomains contribute distinct but essential functions to the Tax response: Tax recognizes the basic region but requires a leucine zipper dimerization domain to increase DNA binding. Tax affects bZIP DNA binding both quantitatively and qualitatively. First, Tax enhances dimerization, thereby increasing the overall level of DNA binding. Second, Tax alters DNA-binding-site selectivity. Both of these effects are probably important for the ability of Tax to recruit the appropriate cellular bZIP protein to the HTLV-I long terminal repeat during viral infection. Recognition of conserved residues within the basic region explains how Tax can increase the DNA binding of a wide variety of bZIPs *in vitro* and activate apparently unrelated viral and cellular promoters *in vivo*. Such 'promiscuous' activation of cellular genes is the probable basis for Tax's oncogenic activity.

bZIP proteins function by forming different combinations of homo- and heterodimers. Recent studies have identified other viral (G.P. and M.R.G., unpublished results) and cellular (ref. 15, and S.W. and M.R.G., in preparation) factors that, like Tax, promote dimerization of sequence-specific DNA-binding proteins. These observations prompt us to speculate that controlling the dimerization of transcription factors may be a common mode of regulation. □

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Mechanism of DNA-binding enhancement by the human T-cell leukaemia virus transactivator Tax

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Tax protein activates transcription of the human T-cell leukaemia virus type I (HTLV-I) genome through three imperfect cyclic AMP-responsive element (CRE) target sites located within the viral promoter¹. Previous work has shown that Tax interacts with the bZIP element of proteins that bind the CRE target site²⁻⁴ to promote peptide dimerization^{3,5}, suggesting an association between Tax and the bZIP coiled coil. Here we show that the site of interaction with Tax is not the coiled coil, but the basic segment. This interaction increases the stability of the GCN4 bZIP dimer by 1.7 kcal mol⁻¹ and the DNA affinity of the dimer by 1.9 kcal mol⁻¹. The differential effect of Tax on several bZIP-DNA complexes that differ in peptide sequence or DNA conformation suggests a model for Tax action based on stabilization of a distinct DNA-bound protein structure. This model may explain how Tax interacts with transcription factors of considerable sequence diversity to alter patterns of gene expression.

Previous work has shown that Tax shifts the bZIP monomer-dimer equilibrium towards the dimer in the absence of DNA and increases the association rate, but not the dissociation rate, of the bZIP-DNA complex³. These data are consistent with two models (Fig. 1). Model 1 invokes a direct interaction between Tax and the bZIP coiled coil that decreases the dissociation constant of the bZIP dimer (K_{dim}); model 2 invokes a direct interaction between Tax and one or more bZIP basic-spacer segments that decreases the dissociation constant of the bZIP-DNA complex, K_{DNA} , as well as K_{dim} . To distinguish model 1 from model 2, we compared the effect of Tax on two GCN4-derived peptides that differed in their ability to form a coiled coil. G_{54} contains the entire GCN4 bZIP element, whereas G_{29}^{SS} contains two copies of the GCN4 basic-spacer segment peptide assembled into a dimer with a disulphide linkage (Fig. 2)^{6,7} and is therefore insensitive to a Tax-induced increase in dimerization. Model 1 predicts that Tax will have no effect on the DNA affinity of G_{29}^{SS} , whereas model 2 predicts that Tax will effect G_{29}^{SS} by an amount corresponding to the effect on DNA binding.

Qualitative electrophoretic mobility shift experiments showed that Tax enhanced DNA binding by both G_{29}^{SS} and G_{54} (Fig. 3). This result demonstrates an interaction between Tax and one or more bZIP basic-spacer segment(s) and provides experimental support for model 2. To determine the relative effects of Tax on dimerization ($\Delta\Delta G_{dim}$) and DNA binding ($\Delta\Delta G_{DNA}$), we measured the effect of Tax on the dissociation constants of the G_{54} -CRE and G_{29}^{SS} -CRE complexes (Fig. 4a). Tax supplemented the binding energies ($\Delta\Delta G_{obs}$) of these two complexes by 3.6 and 1.9 kcal mol⁻¹, respectively. Because G_{54} was predominantly monomeric under the conditions of the DNA titration^{8,9}, the 3.6 kcal mol⁻¹ stabilization with Tax represented the full effect on dimerization and DNA binding. Because G_{29}^{SS} was a covalent dimer, the 1.9 kcal mol⁻¹ stabilization with Tax represented the effect on DNA binding. Taken together, these results apportion

the binding energy supplied to G_{54} by Tax ($\Delta\Delta G_{Tax}^2$) roughly equally between dimerization (1.7 kcal mol⁻¹) and DNA binding (1.9 kcal mol⁻¹)⁵.

We also examined the effect of Tax on a bZIP peptide that, unlike G_{54} , formed a stable coiled-coil dimer. BRCC contained the basic and spacer segments of GCN4 fused to a zipper segment designed to exhibit high helical propensity ($K_{dim} \approx 5$ pM) (ref. 10). Model 1 predicts that the DNA affinity of BRCC will be unaffected by Tax. Model 2, however, predicts that BRCC will be affected by Tax but less than by G_{54} , and this prediction was borne out by experiment: Tax enhanced the stability of the BRCC-CRE complex by 1.0 kcal mol⁻¹ under conditions where the G_{54} -CRE complex was stabilized by 3.1 kcal mol⁻¹ (Fig. 4b).

To identify a set of basic-spacer segment residues important for Tax recognition, we compared the effect of Tax on a series of peptides^{8,9} containing sequences from two bZIP proteins whose homology was restricted to residues essential for DNA recognition (GCN4 and CRE-BP1)^{11,26}. Tax supplemented the binding energies of these peptides by between 1.0 and 3.1 kcal mol⁻¹ under conditions where the binding of G_{54} was enhanced by 3.1 kcal mol⁻¹ (Fig. 4b). The effect of Tax was larger for peptides containing the GCN4 basic segment than for those containing the CRE-BP1 basic segment. However, the effect of Tax on peptides containing the GCN4 basic segment varied depending on the source of the spacer and zipper segments. For example, the CRE target-site affinity of gcg, which contained the basic and zipper segments of GCN4 linked by the spacer segment of CRE-BP1, was enhanced by 2.2 kcal mol⁻¹ (Figs 2 and 4b) ($\Delta\Delta G_{obs} = 3.6$ kcal mol⁻¹). These results indicate that Tax is sensitive to sequence variation throughout the bZIP element⁴.

There are many examples of transcriptional activation through CRE target sites by Tax¹², but few examples of transcriptional activation through AP-1 target sites¹³⁻¹⁵. These two DNA sequences differ by only a single G-C base pair and, owing to an intrinsic major groove bend in the CRE target site, they present similar recognition surfaces in the major groove⁸. To determine whether the qualitative difference in transcriptional activation through CRE and AP-1 target sites by Tax is correlated with the quantitative difference in stabilization of the corresponding bZIP-DNA complexes, we measured the effect of Tax on the CRE and AP-1 target-site affinities of the bZIP peptide ATF-2₃₅₀₋₅₀₅ (ref. 16). Tax stabilized the ATF-2₃₅₀₋₅₀₅-CRE complex by 2.5 kcal mol⁻¹ ($\Delta\Delta G_{Tax}^2$) under conditions where it stabilized the ATF-2₃₅₀₋₅₀₅-AP-1 complex by 1.0 kcal mol⁻¹. Thus Tax increases the selectivity of ATF-2₃₅₀₋₅₀₅ for the CRE target site by 1.5 kcal mol⁻¹. These results suggest that transcriptional activation through CRE target sites by Tax could be favoured because of selective stabilization of bZIP-CRE complexes. Moreover, the observation of different effects of Tax on two bZIP-DNA complexes that differ by a base pair and the conformation of DNA in the complex⁸, combined with our inability to identify a set of bZIP protein side chains essential for Tax activity, support a model in which Tax stabilizes a distinct DNA-bound protein structure.

There are two general mechanisms by which Tax could improve both dimerization and DNA binding. Tax could orient the two basic-spacer segment helices as they emerge from the dimer interface into a conformation appropriate for DNA binding. Recognition of the dimer enhances dimerization; the enhancement in DNA binding results from a reduction of the entropy required to orient the helices. Alternatively, Tax could stabilize helical structure within a single basic-spacer segment, a region that is largely unfolded in the absence of DNA^{6,7,10,17}. Increased helicity within the basic-spacer segment enhances DNA binding; the lowered energetic cost of initiating helical structure enhances dimerization. Tax contains several sequences compatible with the formation of type-II polyproline helices that stabilize α -helical structure in certain small proteins^{18,19} and may act in an intermolecular fashion to stabilize helical structure within the basic-spacer segment. The energetic models presented

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FIG. 1 Two limiting models to account for the improvement in DNA-binding activity of bZIP proteins in the presence of HTLV-I Tax. Shown are reaction coordinate diagrams illustrating the relative free energies of the unfolded bZIP monomer (P), the bZIP dimer (P_2), and the bZIP-DNA complex (P_2 -D) in the presence and absence of Tax (T). The number of Tax molecules represented by T was not determined. **a**, In model 1, Tax interacts with the bZIP coiled coil to decrease the dissociation constant of the dimer (K_{dim}). The improvement in DNA affinity ($\Delta\Delta G_{Tax}^1$) results from a decrease in the energy of activation associated with dimerization (case 1, lower left; dimerization is rate-limiting) or from a shift in the monomer-dimer equilibrium that decreases the energy of activation associated with DNA binding (case 2, lower right; DNA binding is rate-limiting; we assume that $\Delta\Delta G_{dim} = \Delta\Delta G^{1+}$). The absence of an effect of Tax on the dissociation rate of the bZIP-DNA complex³ requires that the increase in apparent binding energy ($\Delta\Delta G_{Tax}^1$) equals the decrease in the energy of the transition state ($\Delta\Delta G^{1+}$). **b**, In model 2, Tax interacts with the bZIP basic-spacer segment (or a pair of basic-spacer segments) to decrease the dissociation constant of the bZIP-DNA complex (K_{DNA}) as well as K_{dim} . The improvement in DNA affinity ($\Delta\Delta G_{Tax}^2$) results from a decrease in the energy of activation associated with dimerization (case 1, lower left) or DNA binding (case 2, lower right) with no decrease in the energy of activation associated with dissociation of the bZIP-DNA complex. In this case, the improvement in apparent binding energy ($\Delta\Delta G_{Tax}^2$) that accounts for the increase in affinity represents the sum of the effects of Tax on dimerization ($\Delta\Delta G_{dim}$) and DNA binding ($\Delta\Delta G_{DNA}$). Although it is unlikely that two bZIP monomers and Tax assemble into a ternary complex in a single step, the kinetic binding pathway is not known. For this reason we depict formation of the Tax-bZIP dimer complex with a single equilibrium.

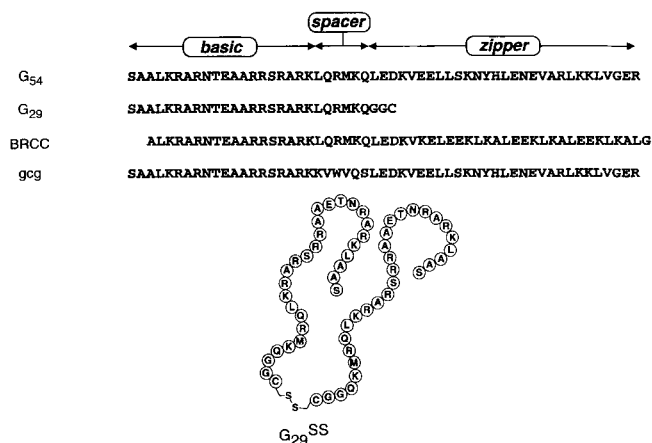
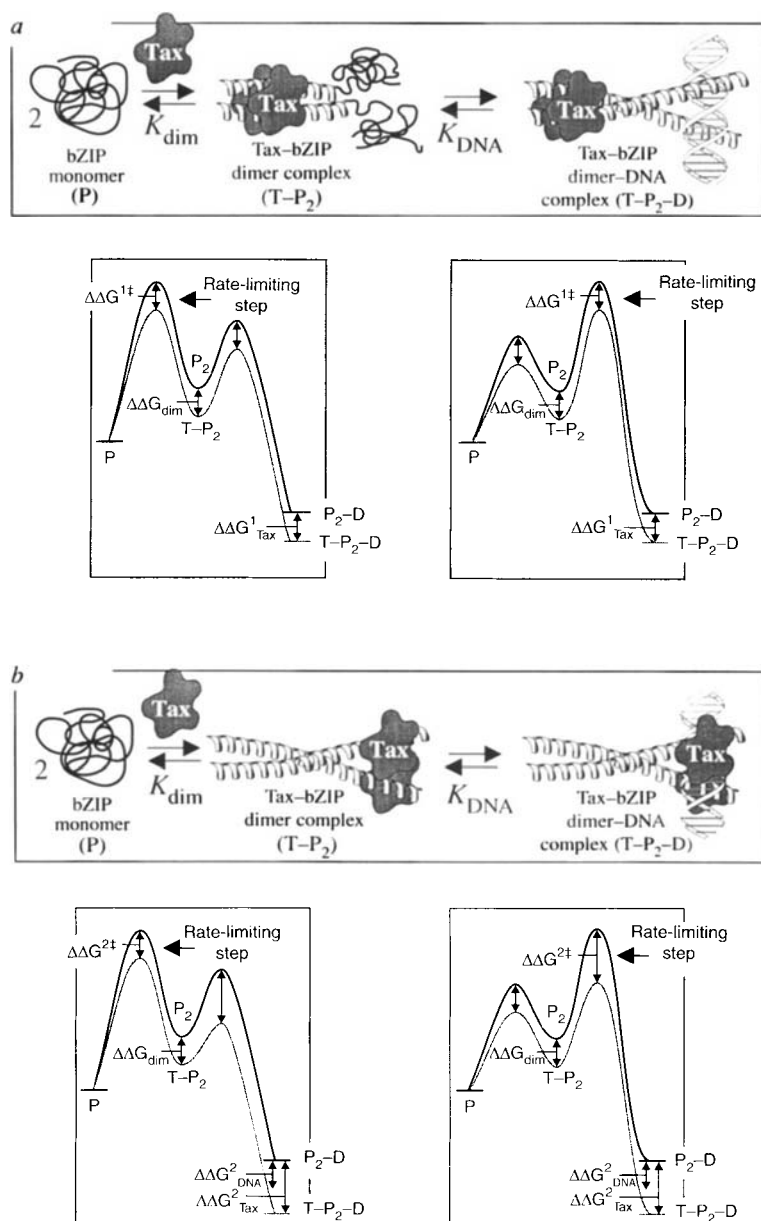
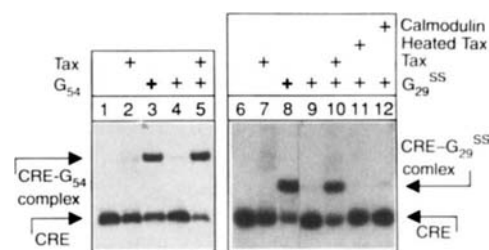


FIG. 2 bZIP peptides used in this study^{8,9}. The bZIP element is composed of a basic segment whose residues participate in DNA contacts^{11,26} joined through a non-conserved six-residue spacer to a zipper segment responsible for protein dimerization²⁴.

FIG. 3 Qualitative analysis of the binding of a 24-base-pair CRE probe and G_{29}^{SS} (ref. 6) or G_{54} (ref. 8). In the absence of Tax (lanes 3 and 8), G_{29}^{SS} and G_{54} showed half-maximal binding at peptide concentrations of 2 and 0.5 nM, respectively. At a concentration of 0.15 nM G_{54} , where little CRE probe was bound in the absence of Tax (lane 4), the addition of 0.3 μ M Tax had a significant effect on the fraction of DNA bound at equilibrium (lane 5). Similar results were obtained with G_{29}^{SS} ; although little CRE probe was bound at a G_{29}^{SS} concentration of 25 pM (lane 9), a significant enhancement was observed in the presence of 0.3 μ M Tax (lane 10). This enhancement in binding was not seen when Tax was denatured by heating in the presence of phenanthroline (lane 11), nor when the reaction was supplemented with an equivalent concentration of calmodulin (lane 12), nor with a mixture of the molecular chaperonin GroEL, adenosine triphosphate and Mg^{2+} in place of Tax.

METHODS. Tax was expressed in *Escherichia coli* from a pTaxH₆ expression vector²⁵ and purified to homogeneity by Ni-chelate chromatography and FPLC (Superdex). Binding reactions contained the indicated peptide and <50 pM of end-labelled CRE probe⁶ (AGTGAGATGACGTCATCTC-



GTGC) in a final reaction mixture containing 75 mM HEPES (pH 7.1), 60 mM KCl, 5 mM $MgCl_2$, 4 μ M $ZnSO_4$, 400 μ M EDTA, 0.5 μ g μ l⁻¹ BSA, 0.5 ng μ l⁻¹ poly(dI-dC)-poly(dI-dC), 4 mM β -mercaptoethanol, 5 ml l⁻¹ Nonidet-40, 100 ml l⁻¹ glycerol (final volume 10 μ l).

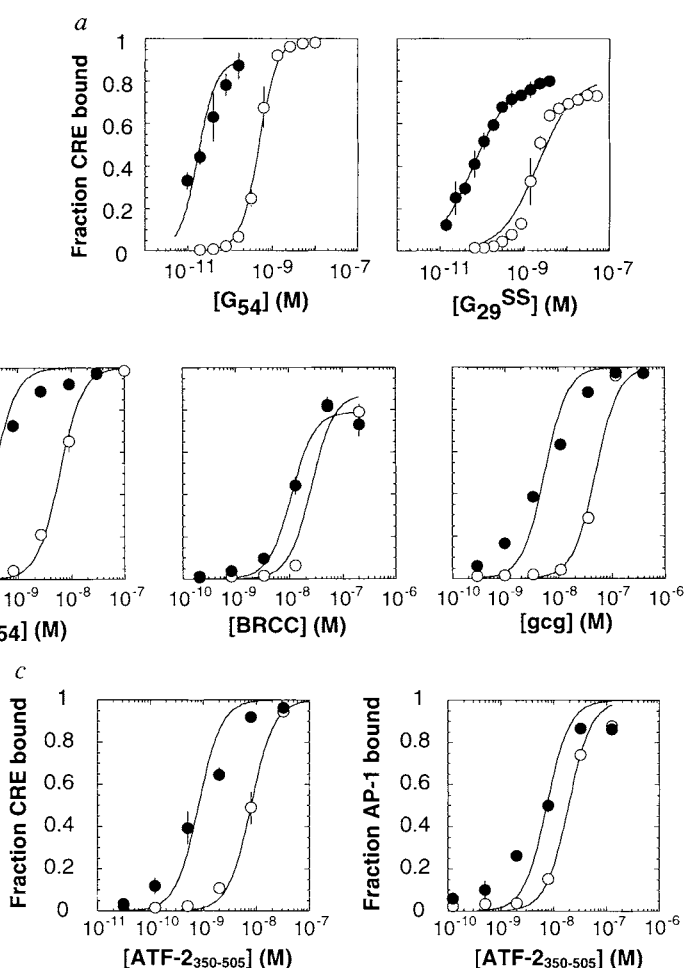
FIG. 4 Quantitative electrophoretic mobility shift analysis of bZIP-DNA complex stability in the presence (filled circles) and absence (open circles) of Tax. **a**, Binding of the CRE probe by G_{54} and G_{29}^{SS} in the presence and absence of 5 μ M Tax. In the absence of Tax, the CRE probe was bound by G_{54} with an apparent dissociation constant (K_{app}) of $(2.3 \pm 0.2) \times 10^{-19}$ M² and by G_{29}^{SS} with an apparent dissociation constant of $(2.1 \pm 0.4) \times 10^{-9}$ M. In the presence of Tax, the dissociation constant of the G_{54} -CRE complex was $(3.3 \pm 1.4) \times 10^{-22}$ M², whereas the dissociation constant of the G_{29}^{SS} -CRE complex was $(6.3 \pm 0.3) \times 10^{-11}$ M. **b**, Analysis of the binding of the CRE probe by G_{54} , BRCC and gcg in the presence and absence of 0.62 μ M Tax. Apparent dissociation constants in the absence of Tax were: G_{54} -CRE, $(3.8 \pm 0.4) \times 10^{-17}$ M²; BRCC-CRE, $(6.8 \pm 3.4) \times 10^{-16}$ M²; gcg-CRE, $(2.3 \pm 0.5) \times 10^{-15}$ M². Apparent dissociation constants in the presence of Tax were: G_{54} -CRE, $(1.3 \pm 0.5) \times 10^{-19}$ M²; BRCC-CRE, $(1.2 \pm 0.3) \times 10^{-16}$ M²; gcg-CRE, $(4.6 \pm 1.8) \times 10^{-17}$ M². **c**, Analysis of the binding of the CRE and AP-1 probes by ATF-2₃₅₀₋₅₀₅ in the presence and absence of 0.62 μ M Tax. Apparent dissociation constants in the absence of Tax were: ATF-2₃₅₀₋₅₀₅, $(6.3 \pm 0.7) \times 10^{-17}$ M²; ATF-2₃₅₀₋₅₀₅, $(3.6 \pm 0.7) \times 10^{-16}$ M². Apparent dissociation constants in the presence of Tax were: ATF-2₃₅₀₋₅₀₅, $(6.6 \pm 3.2) \times 10^{-19}$ M²; ATF-2₃₅₀₋₅₀₅, $(5.5 \pm 2.4) \times 10^{-17}$ M².

METHODS. Binding reactions were performed under conditions similar to those in Fig. 3 except that the final reaction mixture for **b** and **c** contained 80 mM HEPES, pH 7.1, 90 mM KCl, 7.5 mM $MgCl_2$, 6 μ M $ZnSO_4$, 140 ml l⁻¹ glycerol, 0.7 ml l⁻¹ Nonidet 40, 2.4 mM β -mercaptoethanol, 0.1 mg ml⁻¹ BSA and 0.5 ng μ l⁻¹ poly(dI-dC)-poly(dI-dC). Equilibrium dissociation constants of peptide-CRE complexes were obtained by fitting the binding data to the Langmuir equation $\Theta = (S) \times 1 / (1 + K_{app} / ([peptide]_n))$, where K_{app} and S are adjustable parameters ($n = 1$ for G_{29}^{SS} ; $n = 2$ for G_{54} , BRCC, gcg, ATF-2₃₅₀₋₅₀₅) and Θ = fraction of DNA bound. Values for ΔG_{obs} were calculated from the relationship $\Delta G_{obs} = -RT \ln (1/K_d)$, where $R = 0.00198$ kcal mol⁻¹ K⁻¹ and $T = 277$ K. Values for $\Delta \Delta G_{obs}$ are valid with the assumption that Tax does not alter the standard state, as defined by the binding conditions in the absence of Tax.

here provide a framework for analysing the mechanisms by which other accessory factors²⁰⁻²³ interact with bZIP proteins to alter patterns of gene expression. □

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Model for binding of transcription factor TFIIB to the TBP-DNA complex

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TRANSCRIPTION factor TFIIB is essential for the formation of RNA polymerase II initiation complexes where it binds to the TATA-binding protein (TBP) complex with DNA and recruits RNA polymerase II. TFIIB is probably a target for various activators¹⁻⁴. Several models have been proposed for the position of TFIIB in the TFIIB-TBP-DNA complex^{1,3,5,6}. Here we examine the structure of this complex using gel mobility-shift assays and hydroxyl-radical footprinting. TFIIB requires at least seven base pairs of

DNA on either side of the TATA box to form a stable TFIIB-TBP-DNA complex. The sugar residues protected from hydroxyl-radical cleavage by the TFIIB-TBP complex were mapped on the crystal-structure model of the TBP-DNA complex. This analysis suggests that TFIIB binds beneath the concave surface of TBP, contacting DNA both upstream and downstream of the TATA box. Our model predicts that TFIIB binds close to the C-terminal stirrup of TBP and provides one explanation for why TBP needs to bend DNA.

To determine the minimal DNA required for formation of a TFIIB-TBP-DNA complex, we constructed probes of various lengths from the adenovirus major late promoter (Ad-MLP) (Fig. 1a). Gel mobility shift assays were used to assess the ability of these probes to form stable complexes with the conserved region of yeast TBP (residues 61-240) in conjunction with either wild-type human TFIIB (wtTFIIB), or a deletion mutant lacking residues 4-111 (Δ TFIIB). Δ TFIIB retains its TBP-DNA binding domain, but lacks the ability to recruit RNA polymerase II (refs 7-10). Yeast TBP is homologous to human TBP and can form

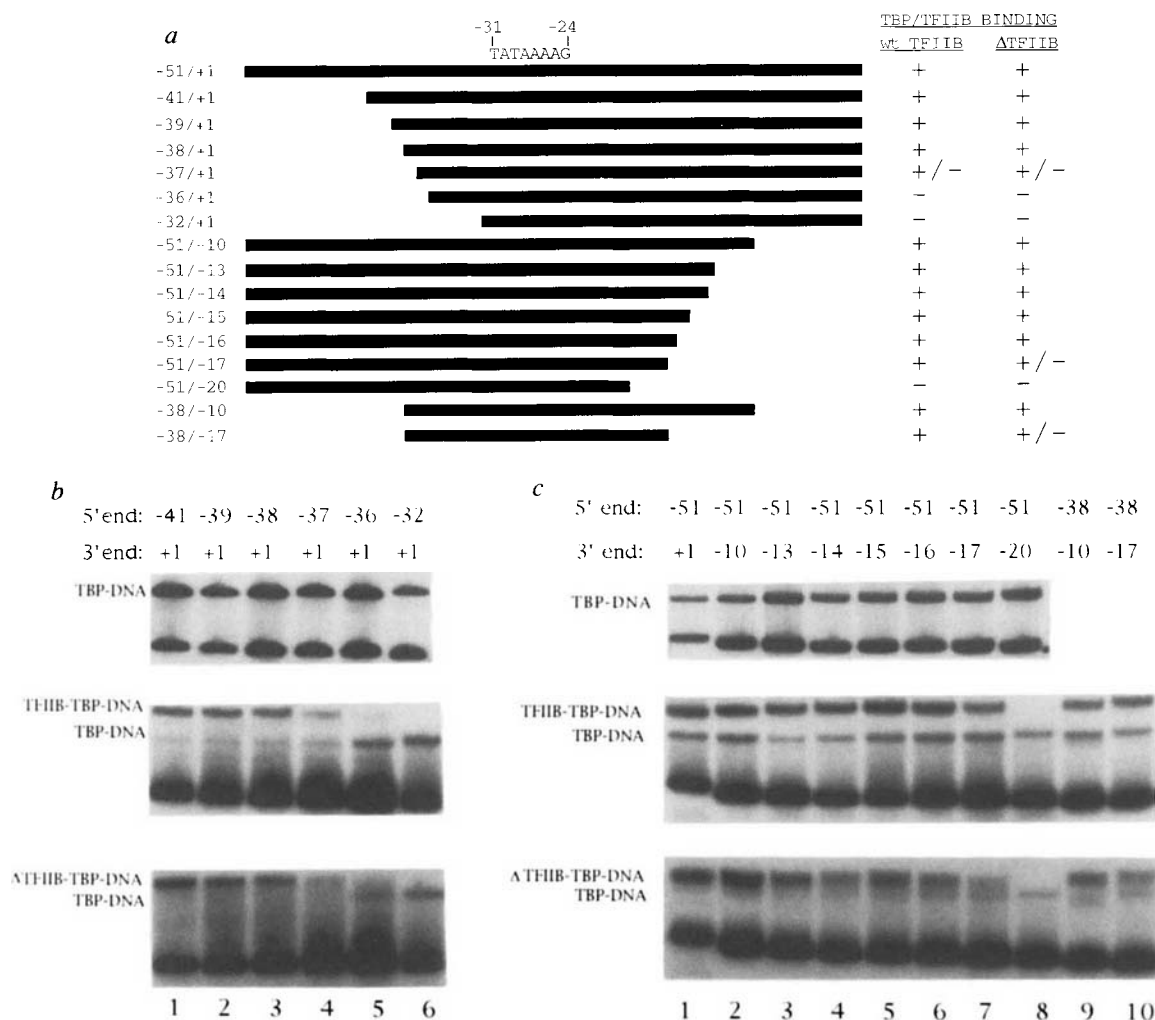


FIG. 1 TFIIB requires DNA upstream and downstream of the TATA box to form a TFIIB-TBP-DNA complex. **a**, Summary of probes assayed and their ability to bind TBP-TFIIB. **b**, Gel mobility-shift assay with DNA probes lacking sequences upstream of TATA. **c**, Gel mobility-shift assay with DNA probes lacking sequences downstream of TATA. Different gel conditions were required to quantify TBP-DNA complexes as these complexes are not optimally stable under the gel conditions required to detect TFIIB-TBP-DNA complexes.

METHODS. Probes were synthesized by polymerase chain reaction (PCR) using plasmid pRW2 (ref. 1), which contains the Ad-MLP from -55 to +33, as a template, and kinase-treated oligonucleotides with indicated 5' and 3' ends. As these probes were synthesized using *Taq* polymerase,

approximately 80% of each probe contains an additional residue added to the 3' end²³. Binding reactions were performed for 45 min as described²⁴, except that gel and running buffers for TBP-DNA and TFIIB-TBP-DNA binding reactions contained 2.5 mM or 1 mM magnesium acetate, respectively. For all binding reactions, the indicated probes (0.01 ng) were incubated with 5 ng of conserved yeast TBP¹⁵ (residues 61-240) in conjunction with 50 ng wtTFIIB or 5 ng Δ TFIIB. Higher concentrations of wtTFIIB were required compared with Δ TFIIB in our reactions, but the specific activities of our proteins have not been determined. Purified recombinant wtTFIIB and Δ TFIIB proteins were a gift from D. Reinberg and provided by Y. Kim.

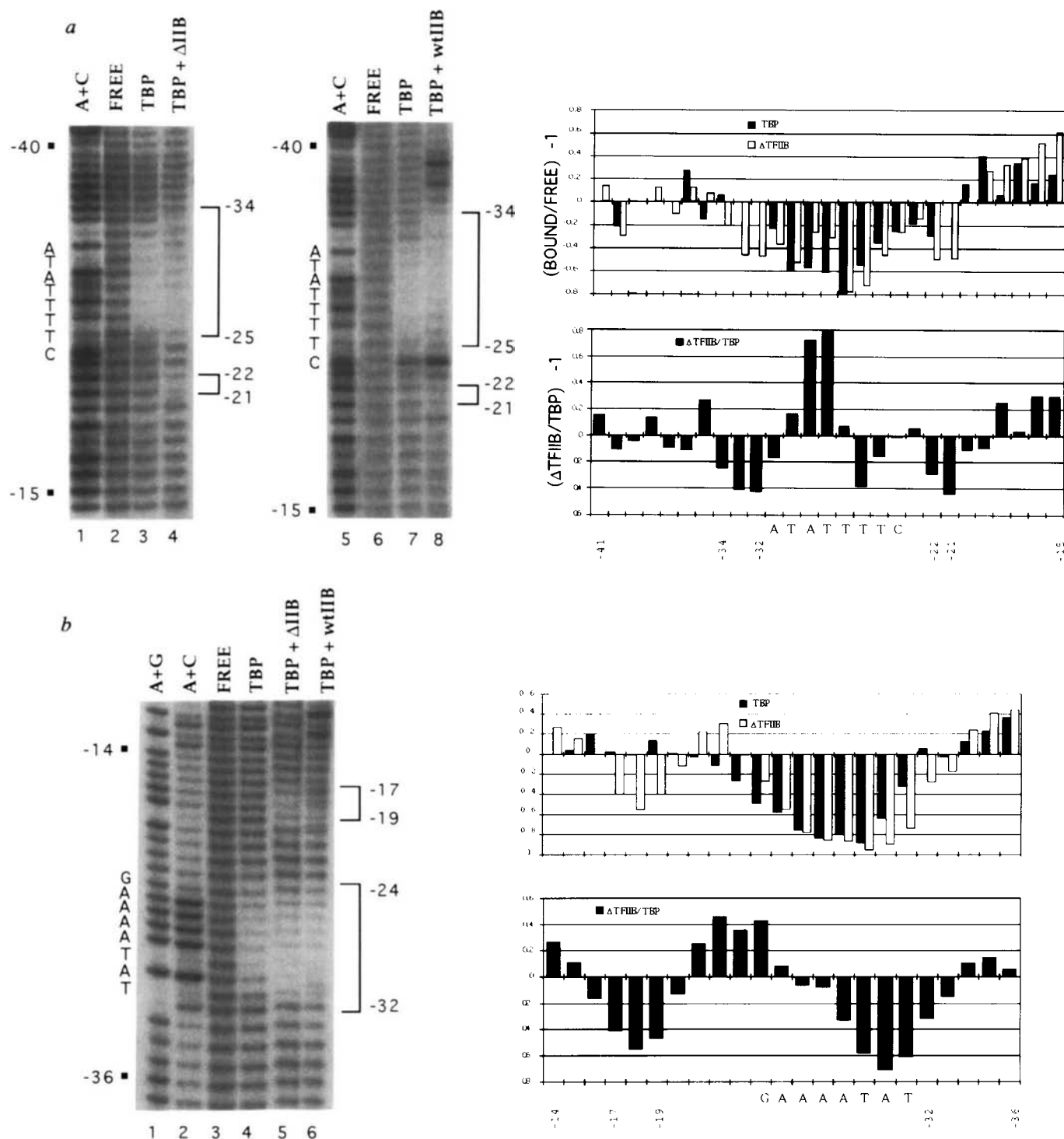


FIG. 2 Hydroxyl-radical protection. *a*, Protection of Ad-MLP transcribed strand. *b*, Protection of Ad-MLP non-transcribed strand. Reactions contained either no protein, 24 ng of conserved yeast TBP alone, or 24 ng of conserved yeast TBP in conjunction with 100 ng wtTFIIB or 20 ng Δ TFIIB. Those residues that are protected from hydroxyl-radical attack in Δ TFIIB-TBP complexes are bracketed. The intensity of individual bands in each reaction was quantified and normalized to its intensity in free DNA. The ratios of the intensities of the bands produced by Δ TFIIB-TBP and by TBP alone were compared for each position (right). Positions showing a decrease in intensity upon binding of proteins are presented as negative values and those showing an increase are shown as positive. Protection by Δ TFIIB was defined as >25% decrease in intensity compared with TBP bound alone.

METHODS. Hydroxyl-radical cleavage reactions were carried out by adding 6 μ l of cleavage solution containing 8.3 mM $\text{Fe}(\text{NH}_4)_2 (\text{SO}_4)_2$,

16.7 mM EDTA, 18.7 mM ascorbic acid, and 2.7% H_2O_2 to 20 μ l protein-binding reactions. Conditions for binding reactions were as for Fig. 1, but without glycerol. Cleavage was for 2 min before addition of 2 μ l 50% glycerol; reactions were then loaded onto polyacrylamide gels (Fig. 1 legend). Protein-DNA complexes were isolated from gels, and the DNA was eluted into 500 μ l 10 mM Tris (pH 8), 1 mM EDTA, and 1 M NaCl buffer. DNA was ethanol-precipitated, resuspended in 4 μ l formamide, and loaded onto an 8% denaturing polyacrylamide gel. Probes were prepared by PCR as for Fig. 1, using kinase-treated oligonucleotides encompassing the Ad-MLP from -55 to +33; all reactions contained 100,000 c.p.m. (0.1 ng) of probe. Quantification was done by Phosphorimager analysis. The ratios of intensities of bands observed for each reaction were normalized using positions outside the protected region.

transcriptionally competent preinitiation complexes with human factors¹.

As expected, we found that TBP bound comparably well with all probes as each contained an intact TATA box (bases -31 to -24). In contrast, only probes containing at least seven bases of DNA upstream of the TATA box could form stable TFIIB-TBP-DNA complexes. This was observed using wtTFIIB as well as Δ TFIIB (Fig. 1b; compare lanes 1-3 with 4-6). Likewise, only probes containing at least seven bases downstream of the TATA box could form stable TFIIB-TBP-DNA complexes (Fig. 1c). To verify that bases -38 to -17 of the Ad-MLP are sufficient for formation of a TFIIB complex, we constructed two short probes consisting of bases -38 to -10 and bases -38 to -17. As expected, these probes were able to form stable TFIIB-TBP-DNA complexes (Fig. 1c, lanes 9 and 10). Although Δ TFIIB formed a slightly less stable complex with DNA ending at -17 than did wtTFIIB (Fig. 1c, lanes 7 and 10), the DNA required for binding full-length or truncated proteins is similar.

To determine the DNA residues contacted by TFIIB, we did hydroxyl-radical footprinting on TFIIB-TBP-DNA complexes. Hydroxyl radical attacks the sugar residues in the DNA backbone¹¹. TBP protected most residues in the Ad-MLP TATA box from hydroxyl-radical attack (Fig. 2a, lanes 3 and 7; b, lane 4)^{12,13}. Addition of Δ TFIIB to TBP-DNA complexes extended the hydroxyl-radical footprint upstream to residue -34, as well as protecting residues -21 and -22 on the transcribed strand of the Ad-MLP (Fig. 2a, lane 4). Δ TFIIB extended the hydroxyl-radical footprint to residue -32 and protected residues -17 to -19 on the non-transcribed strand (Fig. 2b, lane 5). No hydroxyl radical footprints were observed when TBP was omitted in our reactions (not shown).

The hypersensitive cleavages seen in lane 8 of Fig. 2a and lane 6 in Fig. 2b are produced only when wtTFIIB is added to our reactions, are independent of TBP, and require only hydrogen peroxide as a reagent. Thus we believe that these cleavages result from a low level of nonspecific binding of TFIIB to DNA, which facilitates a nucleophilic attack on the DNA backbone by peroxide anion¹⁴ (P. Hopkins and T. Tullius, personal communication). Quantitative analysis of hydroxyl-radical protection by wtTFIIB was complicated by these hypersensitive cleavages, but the footprint produced by TBP-wtTFIIB appears to be similar to the TBP- Δ TFIIB footprint.

As the crystal structure of TBP bound to DNA has been solved^{15,16}, we mapped the sugars protected by Δ TFIIB from hydroxyl-radical attack on the model of the yeast TBP-DNA structure (Fig. 3: top, front view; bottom, back view). The sugars outside the TATA box that are protected upon addition of Δ TFIIB are shown in red, and the eight bases of the TATA box contacted by TBP are blue. In this model, the DNA both upstream and downstream of the 8-base-pair TATA box is presumed to be B-form DNA, as seen in the crystal structure of the TBP-DNA complex. One important limitation of our model is that we cannot predict any DNA distortion induced by TFIIB.

One interpretation of our results is that the conserved region of TBP undergoes a conformational change upon binding TFIIB and requires DNA upstream and/or downstream of the TATA box to form a TFIIB-TBP-DNA complex, but we believe this to be unlikely. The crystal structures of the conserved region of TBP both on and off DNA show that apart from a slight twisting of the region connecting the N- and C-terminal subdomains, TBP does not undergo a conformational change upon binding to DNA^{15,18}. Our results show that an additional seven bases of DNA upstream and downstream of the TATA box are required for a TFIIB-TBP-DNA complex. In order for TBP to interact with seven bases of DNA in addition to the TATA box (or even one extra base), the structure of TBP must be radically altered.

We favour a simpler interpretation in which TFIIB directly interacts with DNA both upstream and downstream of the TATA box. As seen from the localization of protected sugars (Fig. 3), TFIIB most likely binds beneath the concave surface

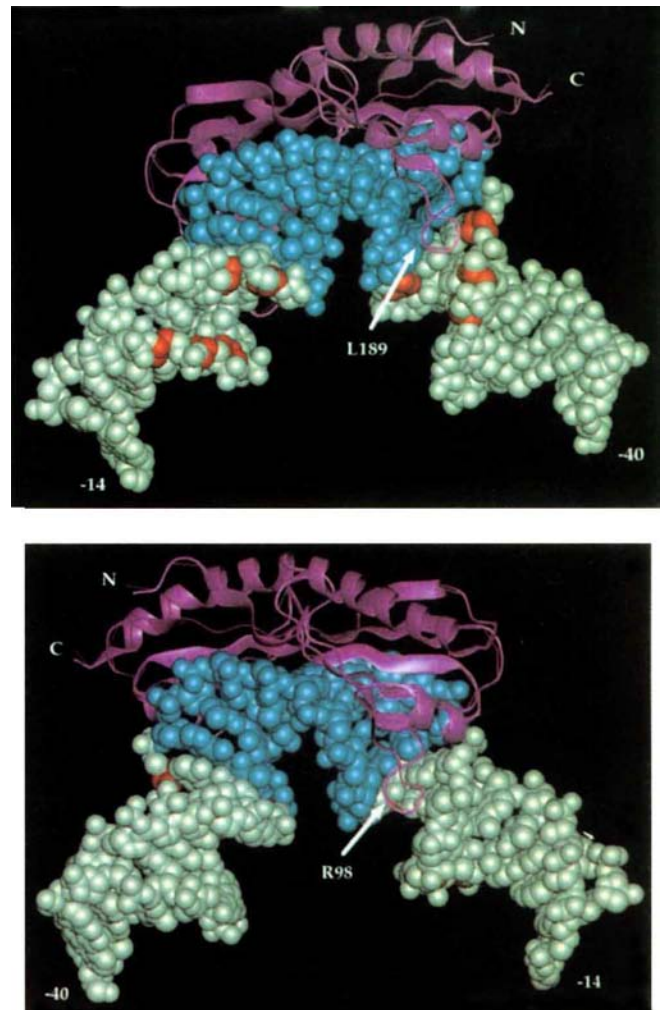


FIG. 3 Structure of TBP-DNA^{15,16} showing sugar residues contacted by TFIIB. Top, front view, and bottom, back view of TBP-DNA complex. The conserved domain of yeast TBP is represented in pink, the eight bases of the TATA box are shown in blue, and sugar residues outside the TATA box that are protected from hydroxyl radical attack by TFIIB are shown in red. Residue 189 in the C-terminal stirrup and the analogous residue 98 in the N-terminal stirrup of TBP are indicated. A mutation in residue 189 alters the ability of yeast TBP to interact with TFIIB. The effect of mutating residue 98 has not been reported.

of TBP, where it contacts DNA primarily on one face of the complex. This is consistent with the weak crosslinking to residue -19 shown by TFIIB⁶ and the DNase I footprint of the TBP- Δ TFIIB-DNA complex⁵. Because of its proximity to contacted base -32, we predict that the C-terminal stirrup of TBP (residues 187-191) is important for TFIIB interaction. In support of this prediction, a mutation in yeast TBP at residue 189 (L189K) abolished the ability of TBP to form stable TFIIB-TBP promoter complexes¹⁹. In addition, alanine substitutions of residues in the C-terminal stirrup of human (h) TBP, but not at any other residue exposed at the surface, block the formation of stable hTBP-TFIIB-DNA complexes (R. Ebright, D. Reinberg, H. Tang and X. Sun, personal communication). Our data predict that the N-terminal stirrup of TBP (residues 96-100) does not contact TFIIB as this stirrup lies on the opposite face, and is distant from sugar residues protected by TFIIB (compare top and bottom in Fig. 3). TFIIB has been shown to crosslink to the first position of the TATA box and with DNA upstream of the TATA box upon irradiation with ultraviolet light⁶, and is proposed to interact with helix H2 of TBP^{20,21}. Thus our results

indicate that TFIIB will lie on the opposite face to TFIIA in the preinitiation complex.

TFIIB exists in solution as a monomer²² and is presumed to bind the TBP-DNA complex as a monomer, but the exact stoichiometry of TFIIB in the TFIIB-TBP complex is not yet known. However, a monomer of the size of the C-terminal domain of TFIIB could interact with both ends of the bent DNA. For comparison, the conserved domain of TBP shown in Fig. 3 is 180 amino acids and the C-terminal domain of TFIIB is 208 amino acids long.

The sugars protected by TFIIB both upstream and downstream of the TATA box border the minor groove in the TBP-DNA model. This suggests that TFIIB, like TBP, interacts at least in part with DNA in the minor groove. Consistent with this prediction, TFIIB binding did not protect any G residues from methylation by dimethyl sulphate at the G+C-rich Ad-

MLP (data not shown). TFIIB does not appear to make essential base-specific contacts with the major groove of the TATA box, as both TBP¹² and TBP-TFIIB form stable complexes with a (dI-dC)-substituted MLP TATA box (data not shown).

Our model for the binding of TFIIB to TBP-DNA revises previous models. Most describe TFIIB interactions with DNA only downstream of the TATA box^{1,3,6}. Our results show that, in addition to the C-terminal stirrup of TBP, a critical element of the TFIIB target is DNA bent in a specific orientation. It is unlikely that a monomer the size of TFIIB could interact with DNA on both sides of the TATA box if the DNA is not bent closer on itself by TBP. TBP may need to bend DNA when it binds in order to support the assembly of preinitiation complexes. TBP bound to distorted DNA is likely to be an important target for other components of the preinitiation complex^{6,15}. □

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Crystal structure of *Thermus aquaticus* DNA polymerase

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THE DNA polymerase from *Thermus aquaticus* (*Taq* polymerase), famous for its use in the polymerase chain reaction, is homologous to *Escherichia coli* DNA polymerase I (pol I) (ref. 1). Like pol I, *Taq* polymerase has a domain at its amino terminus (residues 1-290) that has 5' nuclease activity and a domain at its carboxy terminus that catalyses the polymerase reaction. Unlike pol I, the intervening domain in *Taq* polymerase has lost the editing 3'-5' exonuclease activity. Although the structure of the Klenow fragment of pol I has been known for ten years², that of the intact pol I has proved more elusive. The structure of *Taq* polymerase determined here at 2.4 Å resolution shows that the structures of the polymerase domains of the thermostable enzyme and of the Klenow fragment are nearly identical, whereas the catalytically critical carboxylate residues that bind two metal ions are missing from the remnants of the 3'-5'-exonuclease active site of *Taq* polymerase. The first view of the 5' nuclease domain, responsible for excising the Okazaki RNA in lagging-strand DNA replication, shows a cluster of conserved divalent metal-ion-binding carboxyl-

ates at the bottom of a cleft. The location of this 5'-nuclease active site some 70 Å from the polymerase active site in this crystal form highlights the unanswered question of how this domain works in concert with the polymerase domain to produce a duplex DNA product that contains only a nick.

Crystals of intact *Taq* polymerase (Table 1) diffract to 2.4 Å resolution at -165 °C using synchrotron radiation. The structure was solved initially from a 3.3 Å-resolution electron-density map, phased by multiple heavy-atom isomorphous replacement and improved by solvent flattening using a manually drawn envelope (Fig. 1 and Table 1). Although the polymerase domain shows a 51% amino-acid sequence identity with that of pol I (ref. 1), knowledge of the Klenow fragment (KF) structure did not help in the early stages of phasing, because even this conserved portion contributed too small a fraction to the X-ray scattering. The coordinates of *Taq* polymerase have been partially refined to an *R*-factor of 22.9% (*R*_{free} = 32.2%), with r.m.s. bond and angle deviations of 0.011 Å and 1.79°, respectively, for all data between 10 and 2.4 Å resolution. The tip of the 'thumb' in the polymerase domain is disordered and there are several regions in the 5' nuclease domain where the electron density is discontinuous, presumably because of disordering of loops: these regions include residues 12-13, 68-89, 151-172 and 199-202. Furthermore, the residues from 172 to 233 are built here as polyalanine, again for reasons of poorly ordered electron density. The strung-out arrangement of the three domains in *Taq* polymerase results in an unusually elongated molecule that is 130 Å long in this crystal form (Fig. 2a).

Comparison of the structure of KF with the corresponding parts of the *Taq* polymerase structure shows, as expected from the sequence comparisons, that the polymerase domains are very nearly identical, whereas the 3'-5' exonuclease domains differ extensively (Fig. 2). Least-squares superposition of 353 of 407 corresponding α -carbon atoms in the polymerase domains resulted in an r.m.s. difference of 1.2 Å. By contrast, only 101 of 194 α -carbon atoms in the KF 3'-5' exonuclease domain could

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TABLE 1 Experimental X-ray data and heavy-atom refinement

	Resolution (Å)	Completion (%)	No. unique reflections	R_{sym}^* (%)	$R_{\text{diff}}^\dagger$ (%)	Phasing ‡ power	Mean figure of merit
Native I	2.6	80.0	28,179	6.7	—	—	0.686
Native II	2.4	90.0	40,493	4.6	—	—	
10 mM TMLA $\parallel$	2.5	80.0	31,443	6.9	13.4	1.0	
1 mM CH_3HgCl	4.0	99.4	9,814	6.9	8.7	0.85	
Sat. baker's dimercurial	3.0	95.5	22,127	11.4	11.3	0.54	
1 mM K_2PtCl_4	3.3	97.1	17,447	11.8	27.3	0.53	
1 mM K_2PtI_4	4.0	70.2	6,987	17.6	39.7	0.66	
1 mM CH_3HgCl /10 mM TMLA $\parallel$	3.3	92.0	16,541	11.8	24.9	1.10	

Additional data sets were collected that extended to between 4 and 5 Å resolution for all heavy-atom derivatives (data not shown), and their phasing information was combined with the data shown in the table. These supplementary data produced a map with a better-defined solvent boundary and an improved connectivity of the main-chain backbone. Crystals of *Taq* polymerase were grown at 22 °C in hanging drops containing 3 μl protein solution (10 mg ml^{-1} *Taq* polymerase in 18 mM Tris-HCl, pH 8.2, 0.09 mM EDTA, 0.9 mM DTT, 90 mM KCl, 9% (v/v) glycerol and 0.7% (w/v) β -octyl glucoside) and 3 μl reservoir solution (15% (w/v) PEG8000, 60 mM ammonium sulphate, 2 mM DTT, 0.2% (w/v) sodium azide and 100 mM sodium citrate, pH 5.5)²¹. The crystals belong to space group $P3_121$ and have unit cell dimensions of $a=b=108.0$ Å, $c=171.2$ Å, $\alpha=\beta=90^\circ$ and $\gamma=120^\circ$. The presence of one molecule per asymmetric unit gives a crystal volume per protein mass (V_m) of 3.54 Å³ per dalton and a solvent content of 65% by volume²². In order to make heavy-atom derivatives with mercurials, wild-type *Taq* polymerase (no cysteine residues) was mutated by site-directed mutagenesis to introduce three consecutive cysteines at positions 575 to 577. Crystals of this protein were used for all native and derivative data sets. Crystals were flash-frozen at -165 °C after first transferring to a stabilizing solution containing 40 mM sodium citrate, pH 5.5, 10% (v/v) glycerol, 100 mM KCl, 0.4% (w/v) β -octyl glucoside and 31% (w/v) PEG8000 for 24 h, and then to a second stabilizing solution containing 20 mM HEPES, pH 7.4, 10% (v/v) glycerol, 100 mM KCl, 0.4% (w/v) β -octyl glucoside and 33% (w/v) PEG8000 for 36 h. A 2.6-Å native data set collected at BL-6A2 of the Photon Factory was used in the initial survey for heavy-atom derivatives, but not subsequently. Native I and four derivative data sets were collected at the CHESS A1 beam line ($\lambda=0.908$ Å) equipped with a CCD camera detector. The native II data set was collected at the X12C beam line of the National Synchrotron Light Source (NSLS) at the Brookhaven National Laboratory ($\lambda=1.00$ Å equipped with the MAR X-ray detector system). Two derivative data sets were collected on an R-Axis IIC X-ray detector system mounted on a Rigaku-200 rotating anode. All data were reduced using DENZO and scaled using SCALEPACK (programs written by Z. Otwinowski). The position of the *Taq* polymerase in the crystal was solved by molecular replacement at 4 Å resolution using a model of the polymerase domain that contained 45% of the scattering mass of *Taq* polymerases and was based on the structure of Klenow fragment refined at 2.5 Å resolution (J. Jaeger, D. Cahill and T.A.S., unpublished result). The rotation function search was done using MERLOT²³, the Patterson correlation refinement and the translation function in X-PLOR²⁴. Phases calculated from the model allowed location of bound heavy atoms by difference-Fourier syntheses at 4 Å resolution but were not used directly in the structure determination. An MIR electron density map calculated at 3.3 Å resolution using these derivatives refined with ML-PHARE²⁵ was improved by solvent flattening, histogram matching and phase combination using SQUASH²⁶. A polyaniline model fitted to this map allowed computation of a molecular envelope around the model with a 5 Å radius for each atom using the program O (ref. 27). Further solvent flattening with this manually drawn envelope using SQUASH and interactive heavy-atom refinement against flattened phases improved the map quality. Refinement of the structure built into this map, including simulated annealing, position refinement and manual model rebuilding was done against data from 10 to 2.4 Å resolution. The present structure includes 776 amino acids, one β -octyl glucoside, and 297 water molecules. Fifty-six amino acids are not visible and sixty are modelled by polyaniline.

* $R_{\text{sym}} = \sum |I - \langle I \rangle| / \sum I$, where I is the observed intensity and $\langle I \rangle$ is the average intensity from multiple measurements.

† $R_{\text{diff}} = \sum ||F_{\text{PH}}| - |F_{\text{P}}|| / \sum |F_{\text{P}}|$

‡ Data beyond 3.3 Å resolution were not included in the phase refinement of heavy-atom statistics for any derivatives.

§ Phasing power, $F_{\text{H}}/\epsilon = \text{r.m.s.}(F_{\text{H}})/\text{r.m.s.}(\text{lack of closure})$, where F_{H} is the calculated heavy-atom structure factor.

|| TMLA, trimethyl lead acetate. A crystal was soaked in 1 mM CH_3HgCl for 20 h and then transferred to 10 mM TMLA solution to be soaked for 72 h.

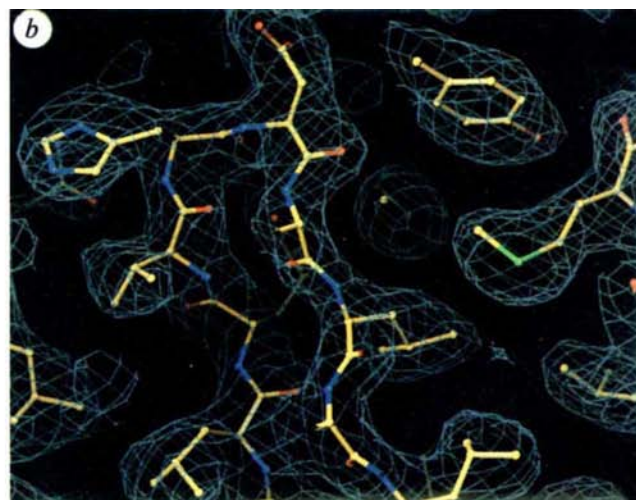
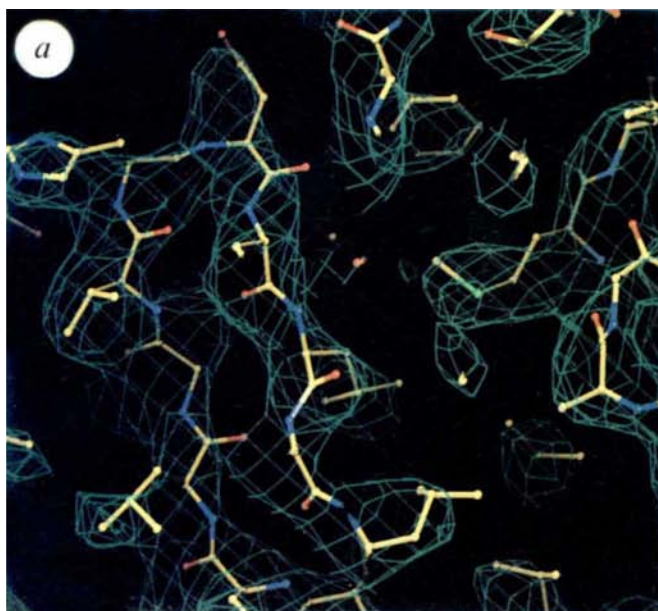
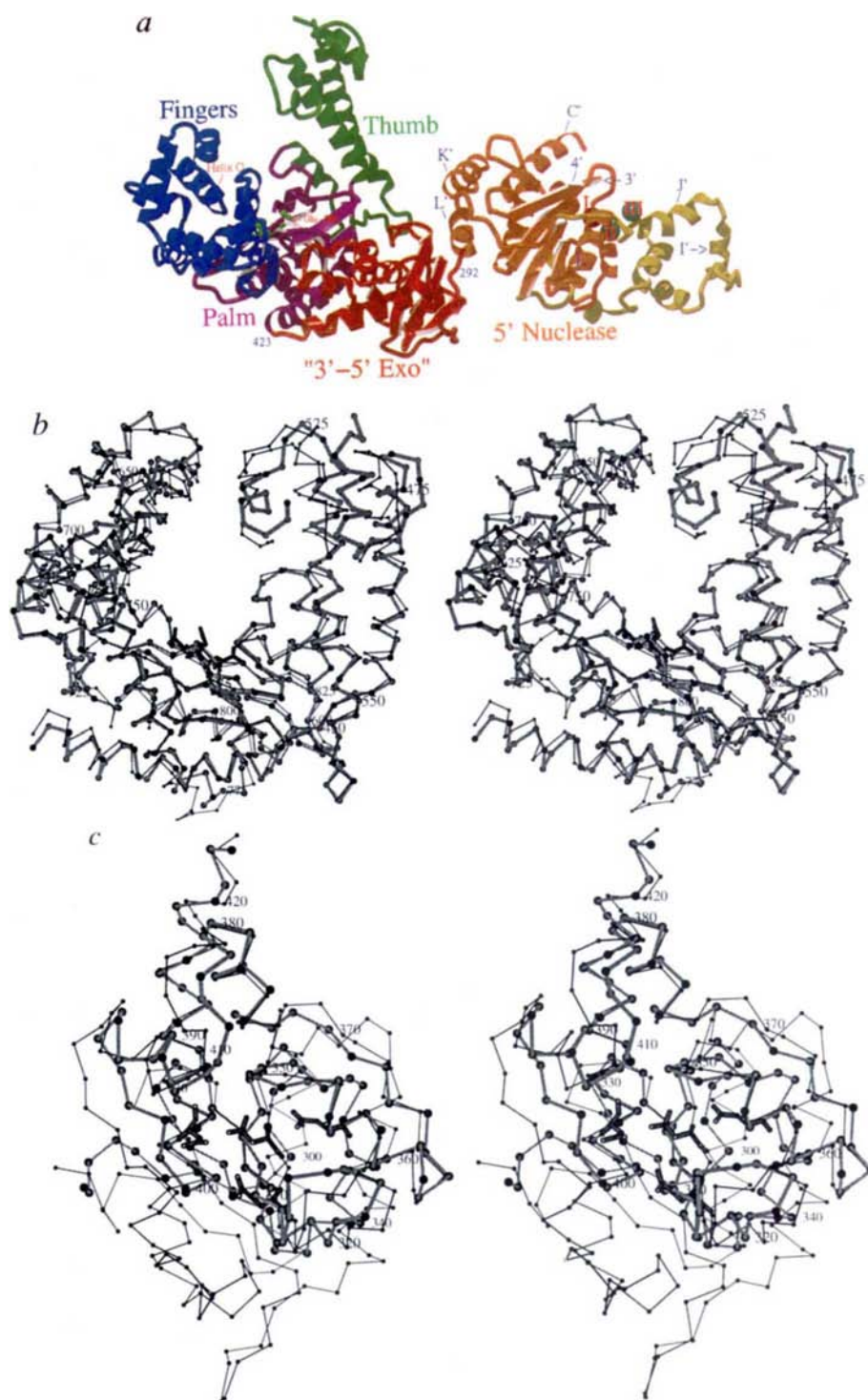


FIG. 1 Electron density maps with the refined model superimposed. *a*, Solvent-flattened multiple isomorphous replacement (MIR) map at 3.3 Å resolution; *b*, 2.4 Å-resolution deletion map calculated using $2F_o - F_c$ as amplitudes and using phases calculated from the structure omitting the portion of the model shown.

FIG. 2 *Taq* polymerase structure and comparison with that of KF. **a**, Helix-and-arrow overall schematic ribbon representation of *Taq* polymerase drawn using rendered MOLSCRIPT^{28,29}. α -Helices are represented as helical ribbons and β -strands as arrows. Helices and strands are lettered and numbered as in KF for the 3'-5' exonuclease and polymerase domains and lettered and numbered with primes in the 5' nuclease (previously called 5'-3' exonuclease) domain. The 5' nuclease domain at the N terminus is orange and yellow; its active site is marked by a red Zn^{2+} and two blue Mn^{2+} ions. The portion of this domain whose side chains have not been positioned is yellow (residues 172-234 and 1-12). The vestigial 3'-5' exonuclease domain is red and the polymerase domain is divided into green thumb, blue finger and purple palm subdomains. The active site Asp 610, Asp 785 and Glu 786 are in dark green. **b**, Superposition of KF and *Taq* polymerase. Stereo of $C\alpha$ backbone of the KF polymerase domain (thin bonds) superimposed on the corresponding atoms (thick bonds) of the *Taq* polymerase, which are numbered. The three catalytic carboxylate side chains are shown in ball and stick representation at the bottom of the cleft. **c**, Superposition of 131 α -carbon atoms of the 3'-5' exonuclease domain of KF (thin bonds) on the corresponding atoms of *Taq* polymerase. The four catalytic carboxylates in KF are shown. **d**, Structure based alignment of the sequences of the 3'-5' exonuclease domain of KF on the corresponding domain of *Taq* polymerase. The unaligned residues are shown as dots in *E. coli* polymerase I (E.c.) and the unpaired missing residues are shown as blank in *Taq* polymerase (T.aq). The amino-acid sequence numbers of *Taq* DNA polymerase secondary structure elements are as follows: 5' nuclease domain: 1'(3-7) 2'(12-17) A'(18-29) B'(42-57) 3'(60-67) C'(91-106) 4'(108-113) D'(119-132) 5'(134-139) E'(143-148) 6'(175-178) F'(179-183) G'(189-198) H'(203-213) h'(217-221) I'(225-232) J'(235-246) K'(266-276) L'(281-289); 3'-5' exonuclease domain: 1(294-298) A(-) 2(305-312) 3(322-328) 4(330-336) B(338-344) 5(347-351) C(353-362) 5a(368-372) D(373-380) E(387-394) F(402-422); polymerase domain: G(424-447) 6(448-452) H(453-477) Ha(487-496) Hb(515-521) I(527-552) 7(559-598) 8(572-578) J(581-584) K(589-598) 9(603-613) L(614-623) M(626-634) N(638-648) O(656-670) Oa(675-723) Ob(688-699) P(702-717) 10(718-723) 11(724-729) Q(740-774) 12(776-784) 13(785-792) R(795-810) 14(816-825) Ra(826-831).



d

T.aq. 292 kaleeapw...eGAFVGFVLSRKEPMADLLALAAARGG 330
E.c. 327 ychvtil...pVFAFDITETDSLINISANVLGLSFATEP 377

T.aq. 331 RVHRAp epykalrdl keaRGLAKDL 356
E.c. 379 VAAYTp...sreraell...alkVGQNLKYD 424

T.aq. 357 SVLAIREGLGLP PGDDPMLAYLLDP SNITPEGVARRYGgewte 400
E.c. 425 RGLIANYGIELR.IAFDITMESYIINS..GRHIMDSIAERWLkkti 471 ...

T.aq. 401 EAGRAALSERLFANLWGRLEGE 423
E.c. 497 YAAEDADVITQLHLKMPDLQKH 519

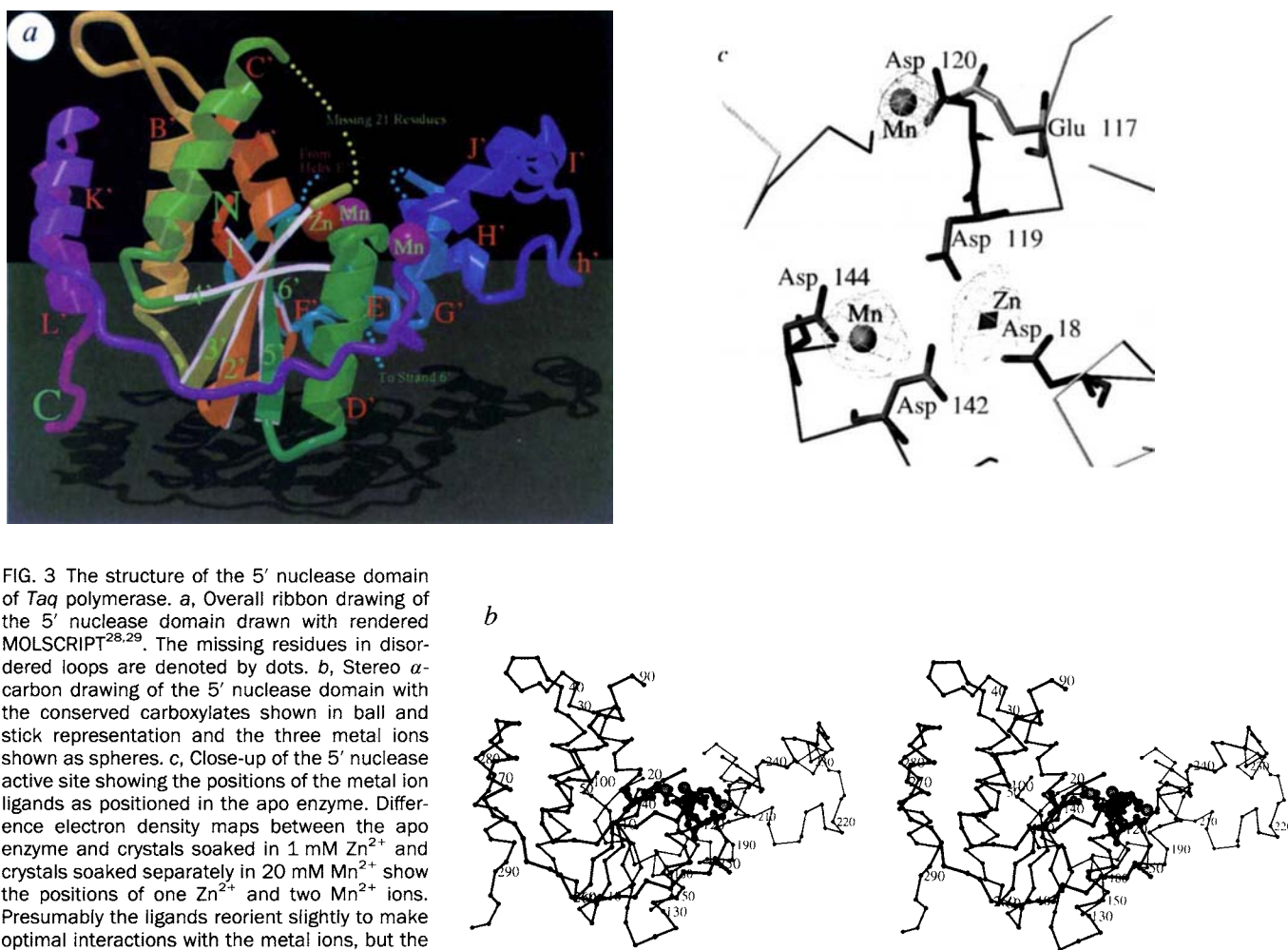


FIG. 3 The structure of the 5' nuclease domain of *Taq* polymerase. **a**, Overall ribbon drawing of the 5' nuclease domain drawn with rendered MOLSCRIPT^{28,29}. The missing residues in disordered loops are denoted by dots. **b**, Stereo α -carbon drawing of the 5' nuclease domain with the conserved carboxylates shown in ball and stick representation and the three metal ions shown as spheres. **c**, Close-up of the 5' nuclease active site showing the positions of the metal ion ligands as positioned in the apo enzyme. Difference electron density maps between the apo enzyme and crystals soaked in 1 mM Zn^{2+} and crystals soaked separately in 20 mM Mn^{2+} show the positions of one Zn^{2+} and two Mn^{2+} ions. Presumably the ligands reorient slightly to make optimal interactions with the metal ions, but the complex structures have not yet been refined.

be superimposed on the corresponding atoms in the 131-residue domain in the *Taq* enzyme to give an r.m.s. difference of 1.6 Å. One major difference in the overall structure of the 3'-5' domain of *Taq* polymerase as compared with that of KF is the deletion of four loops of lengths between 8 to 27 residues. In KF, these loops pack together on one side of the 3'-5' exonuclease domain (Fig. 2b). Furthermore, all four of the carboxylates (D424, D501, D355, E357) known to be essential for divalent metal binding and catalysis in the 3'-5' exonuclease domain of KF^{3,4} have been replaced by residues incapable of binding metal ions (L356, R405, G308, V310) in the vestigial 3'-5' exonuclease domain of *Taq* polymerase. Although the 3'-5' exonuclease catalytic site has been destroyed and the size of the domain reduced, the contact with the pol domain and the distance between the polymerase and 3'-5' exonuclease domains remains similar in the two homologous polymerases.

The 5'-nuclease domain forms a structure that is separate from the other two domains (Fig. 2), with only 850 Å² of surface area in contact with the 3'-5' exonuclease domain, consistent with this domain's ability to function after its proteolytic removal from the rest of the protein (J. E. Dahlberg, personal communication, and ref. 5). It has a deep cleft that contains at its bottom the conserved carboxylates shown here to ligate divalent metal ions. A central β -sheet lies at the heart of the domain and is flanked on both sides by assemblies of five and six α -helices which form the walls of the active-site cleft (Fig. 3).

Alignment of the amino-acid sequences of six 5' nuclease domains from DNA polymerases in the pol I family show six highly conserved sequence motifs containing ten conserved acidic residues⁶. Seven of these residues (Asp 18, Asp 67,

Glu 117, Asp 119, Asp 120, Asp 142 and Asp 144) cluster within a sphere of 7 Å radius, two (Asp 188 and Asp 191) lie in a region built as polyalanine, and one (Glu 76) occurs in a completely disordered loop. Crystallographic data from crystals soaked in divalent metal ions show that some of these carboxylates serve to ligate as many as three divalent metal ions (Fig. 3c). A difference Fourier using data from crystals soaked in 20 mM Mn^{2+} shows two peaks, corresponding to a metal-ion site III that is interacting with Glu 117, Asp 120, and possibly Asp 119, and a metal-ion site II that is interacting with Asp 142 and Asp 144. Soaking crystals in 1 mM Zn^{2+} reveals a divalent metal-ion-binding site I, whose ligands appear to be Asp 18, Asp 119, Asp 142 and perhaps His 21. Electron density maps of the apo protein show some density at metal-ion site I, which may indicate binding of a partially substituted Zn^{2+} ion. Sites I and II are separated by about 5 Å, whereas these two sites are each about 10 Å from site III.

As we do not yet have the structures of either substrate or product complexes with the enzyme, a firm mechanism for 5'-nuclease reaction⁷ cannot be proposed. However, a mechanism of phosphoryl transfer is becoming apparent in an increasing number of enzymes in which two divalent metal ions are involved. This two-metal-ion mechanism was suggested initially for the 3'-5' exonuclease of KF⁸ and is supported by structural, mutagenic and kinetic studies^{3,4,9}. There is evidence that the enzymes alkaline phosphatase¹⁰, pyrophosphatase^{11,12}, RNase H (refs 13, 14) and polymerase, to name a few, use a similar mechanism, as may¹⁵⁻¹⁷ other enzymes containing conserved, catalytically essential carboxylates (such as *ruvC*¹⁸ and HIV integrase¹⁹)²⁰. In this mechanism, the two metal ions are

generally 4 Å apart, interact directly with the scissile phosphate, stabilize the pentacoordinate intermediate, and generate the attacking hydroxide ion, as well as facilitating the departure of the 3' oxyanion⁸. If such a two-metal-ion mechanism is relevant to the 5' nuclease, the possible role, if any, of the more distant site III metal ion can only be guessed.

The source of the thermal stability of *Taq* polymerase is not obvious from structural comparison with KF, but the number of hydrogen bonds has increased by four, and two salt bridges between subdomains in the polymerase domain become hydrophobic; the ratio of leucine to isoleucine has increased by 4.4-fold, and arginine to lysine by 1.3-fold, which may result from the higher G+C content of the leucine and arginine codons (giving a more thermostable DNA), rather than an effect on the protein.

An important question concerning the pol I family of enzymes is how the polymerase and 5'-nuclease active sites work together to generate a duplex DNA product containing only a nick: the present structure raises at least as many questions as it answers, because we observe that these two active sites are separated by over 70 Å. The unusually elongated shape of the molecule seen here led us to examine its overall fold in solution. Preliminary measurements of the radius of gyration (R_g) of *Taq* polymerase by solution X-ray-scattering methods yield an experimental value of R_g that is substantially smaller than that calculated from the coordinates of the crystal structure (S.H.E. *et al.*, unpublished observations). Thus the 5' nuclease domain is not positioned in solution as shown in Fig. 2, but must be located much closer to the centre of mass of the Stoffel fragment. Presumably its orientation in these crystals is adventitious and governed by crystal-packing interactions. Two packing interactions between the 5' nuclease and neighbouring molecules bury 1,100 and 1,466 Å² of solvent-accessible area, larger than the intramolecular interaction surface. A structural basis for understanding how these two activities work together must await the crystal structure of a complex with the appropriately nicked DNA substrate. □

ERRATUM

Crystal structure of a replication fork single-stranded DNA binding protein (T4 gp32) complexed to DNA

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Nature 376, 362–366 (1995)

AN error in the production process resulted in Fig. 1a and b of the paper by Kim *et al.* on page 613 of this issue being substituted for Fig. 2a and b of the earlier paper by Shamoo *et al.* The correct panels of Fig. 2 are shown here.

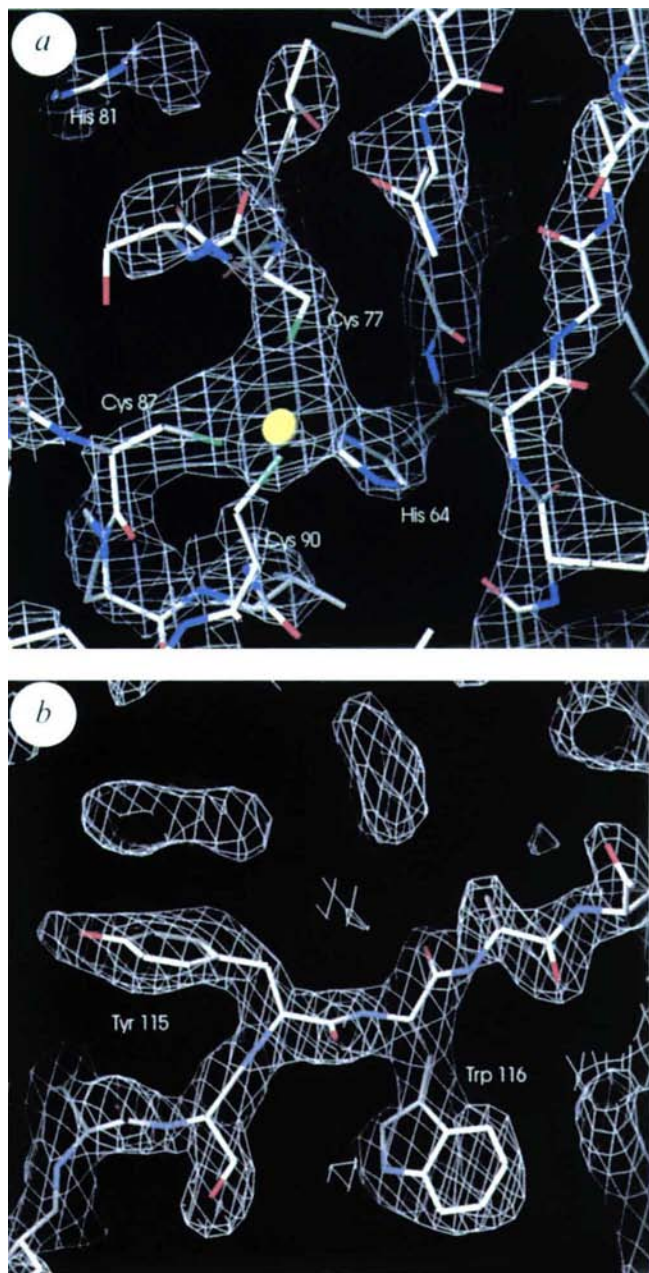


FIG. 2 a, Experimental electron density map to 3.1 Å contoured at 2.5 σ and calculated using the combined MAD and MIR phases that have been solvent flattened. The coordination of the Zn²⁺ ion (yellow) is tetrahedral with His 64, Cys 77, Cys 87 and Cys 90 as ligands. b, 2F_o - F_c electron density map contoured at 1.3 σ showing a stretch of β -strand 4 that includes the current partly refined model and all the data to 2.2 Å.

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The surface force apparatus — a tool for probing molecular protein interactions

Deborah Leckband

Force measurements can probe directly the molecular impact of proteins' electrostatic surface properties, specific bond strengths, membrane composition, and membrane fluidity on biological recognition events.

A FUNDAMENTAL concern of biological research is the correlation of molecular architecture with function¹. A particular long-range goal is the prediction of the molecular interactions that mediate phenomena, such as cell adhesion and binding affinities directly from known molecular structures². The behaviour and properties of small molecules can often be predicted by molecular modeling. On the other hand, the computational requirements of large systems and the paucity of accurate interaction potentials is limiting. Ideally, one would measure the potential fields and intermolecular forces directly.

Such direct measurements are possible with the surface force apparatus (SFA). Initial designs by Tabor and Winterton³ were extensively modified by Israelachvili and Adams⁴ for measurements in liquids. The SFA is now a widely used and powerful instrument used to identify the fundamental forces that operate in both biological and non-biological systems⁵⁻⁷. With this instrument, one measures the magnitude and distance dependence of the molecular forces acting between two surfaces⁸. Forces are measured with a resolution of 10 nN, and the separations are determined *in situ* with ± 1 Å resolution by optical interferometry^{8,9}. Although many instruments have been custom-built, several designs can now be purchased commercially¹⁰.

The sample materials can be in principle anything of interest with the stipulation that the samples are laterally homogeneous and of uniform, controlled thickness^{5,8}. Materials are typically supported on molecularly smooth mica sheets to minimize their roughness. The samples are then mounted in the apparatus on transparent silica lenses (Fig. 1). In protein measurements, the proteins can be physisorbed or covalently

attached to mica^{11,12}, but we typically immobilize and orient them by specific binding to receptor-functionalized planar lipid bilayers supported on mica sheets (Fig. 2)^{13,14}. As the measurements are conducted in aqueous electrolyte solutions, one can determine the effects of ionic strength, pH, and ionic composition¹³⁻¹⁵. The solution composition can also be changed during experiments¹³⁻¹⁵. Together with these experimental variables, the SFA has made possible direct determinations of (1) the molecular properties of and forces exerted by protein structural motifs^{13,14} and (2) the molecular forces and mechanisms that modulate receptor-mediated binding to surfaces^{13,14,16,17}.

Electrostatic properties

In order to probe electrostatic potential maps of protein surfaces, one could determine the theoretical electrostatic response of test

charges near the protein surface². With the SFA, the electrostatic properties of the surfaces of immobilized proteins are determined from the measured forces between protein monolayers and a second 'test' surface (Fig. 2). The 'test' surfaces are typically protein monolayers or receptor-functionalized membranes^{13,14}. Fits of measured intersurface forces to solutions of the nonlinear Poisson-Boltzmann equation give the electrostatic potentials of the protein monolayers^{13,15}. Previous work demonstrated that these measurements probe the local — not global — electrostatic properties of protein surfaces, and that one can detect changes in single charged amino acids^{13,14}. Typical errors in the measured electrostatic potentials are ± 5 mV, which corresponds to ± 0.2 unit charge per protein at 4,500 Å² per protein in 1 mM NaCl (ref. 13). Increased protein surface coverage and/or magnitudes of charge perturbations can minimize errors. The proteins must, however, be oriented uniformly in order to relate measured electrostatic forces to protein structure.

Because of the sample configurations, these fitted electrostatic potentials do not correspond quantitatively to those of the single protein molecules^{13,14,18}. They nevertheless detect directly the influence of local structural motifs on protein electrostatic surface properties and the pH, ionic strength and compositional dependences of those forces^{13,14}. With streptavidin monolayers, for example, the pH dependence of the electrostatic potential of the biotin-binding surface indicates that two histidines control the surface electrostatics between pH 6 and 7.2 (ref. 13). A ring of positive charge surrounding the binding site of an antifluorescein antibody was shown to control the electrostatic potential of the binding surface and its interactions with fluorescein¹⁴. Such measurements demonstrate not only the impact of local

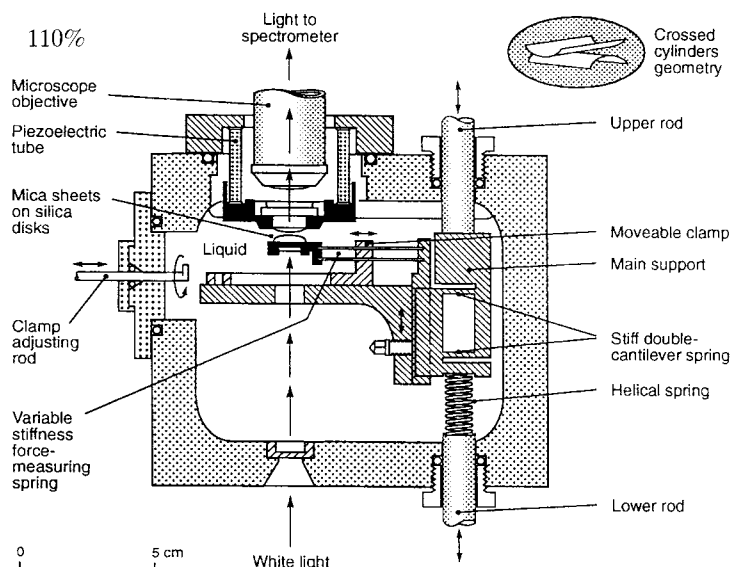


FIG. 1 Schematic of a Mark II model surface force apparatus. The locations and relative orientation of the transparent silica lenses that support the sample-coated mica sheets are as indicated. As the separation distance between the surfaces is decreased, the force at each separation is determined from the measured deflection in the force-measuring spring. The light exiting the interferometric cavity, which contains the samples, is focused onto the slit of a spectrometer. The surface separations are then determined from the resulting interference fringes of equal chromatic order (FECO) observed with the spectrometer. The complete optical system used to analyse the interference fringes is not shown here, but has been described elsewhere⁵. During force measurements with biological materials, the chamber is filled with liquid, the composition of which can be changed during experiments. The temperature of the experiment is controlled externally.

protein structure on electrostatic surface properties, but also the range and the magnitude of the resultant receptor-ligand forces acting along a uniaxial trajectory. Moreover, because relative changes in surface electrostatic potentials correspond to relative changes in the electrostatic potentials of the individual proteins¹⁸, it is possible to determine semiquantitatively the electrostatic potential differences between, for example, charge mutants.

Molecular recognition

In addition to protein structure, complex environments such as cell membranes can also substantially impact protein function^{19,20}. We recently demonstrated the ability to probe the molecular basis of such microenvironmental effects with the SFA¹⁴. For example, from the measured force-distance profiles and adhesion between proteins and immobilized receptors, membrane hydration/undulation was shown to impede substantially protein binding to membrane-bound receptors¹⁴. In particular, a 7 Å increase in the steric hydration barrier thickness decreased antibody adhesion to immobilized antigen by 50 per cent¹⁴. Streptavidin adhesion to biotinylated membranes exhib-

ited a similar hydration-dependent attenuation¹³. Moreover, the incorporation of measured hydration barriers in a hydrodynamic model of streptavidin adsorption to biotin-derivatized optical fibers was necessary to fit experimental data²¹. These studies focused on the subtle effects of small hydration thickness changes, but by construction of appropriate model systems, SFA measurements can also be extended to investigations of other microenvironment effects, such as surface composition or membrane fluidity¹³.

The SFA has also been used to determine the mechanisms that modulate receptor-mediated intermembrane adhesion, that determine the tensile strengths of specific intermembrane cross-bridges. Recently, the rupture force per bond, not the intrinsic bond energy, was shown to determine both the tensile strengths and failure mechanisms of receptor-mediated intermembrane bonds¹⁶. With robust protein-anchoring methods²², the tensile strengths of receptor-ligand bonds can also be measured.

Time-dependent processes

In many cases, the dynamics of biological events at membrane surfaces are of tremendous importance²³. One can readily investi-

gate such time-dependent molecular events *in situ* by creating an interface between the optical system of the SFA and a video camera, recorder and internal timer⁵. The dynamic events observable with this system are limited by camera response times, the magnitudes of the changes and data capture rates. In membrane fusion experiments, for example, fusion-site nucleation and the subsequent hemi-fusion of the two outer monolayers were observed directly and in real-time²⁴. Time-dependent measurements of streptavidin interactions with biotinylated membranes revealed the dynamics of biotin interactions with the streptavidin surface prior to the insertion of biotin into the active site¹³.

Summary

The measurements described demonstrate the variety of protein or protein-mediated interactions that can be probed directly with the SFA. The distance, force and dynamic measurement capabilities provide tremendous potential for the identification and quantification of the molecular mechanisms and forces that both determine protein properties and influence protein function in diverse environments.

Deborah Leckband is in the Department of Chemical Engineering at the University of Illinois at Urbana-Champaign, Urbana, Illinois 61801 USA. For more information, fill in reader service number 100 on the reader service card.

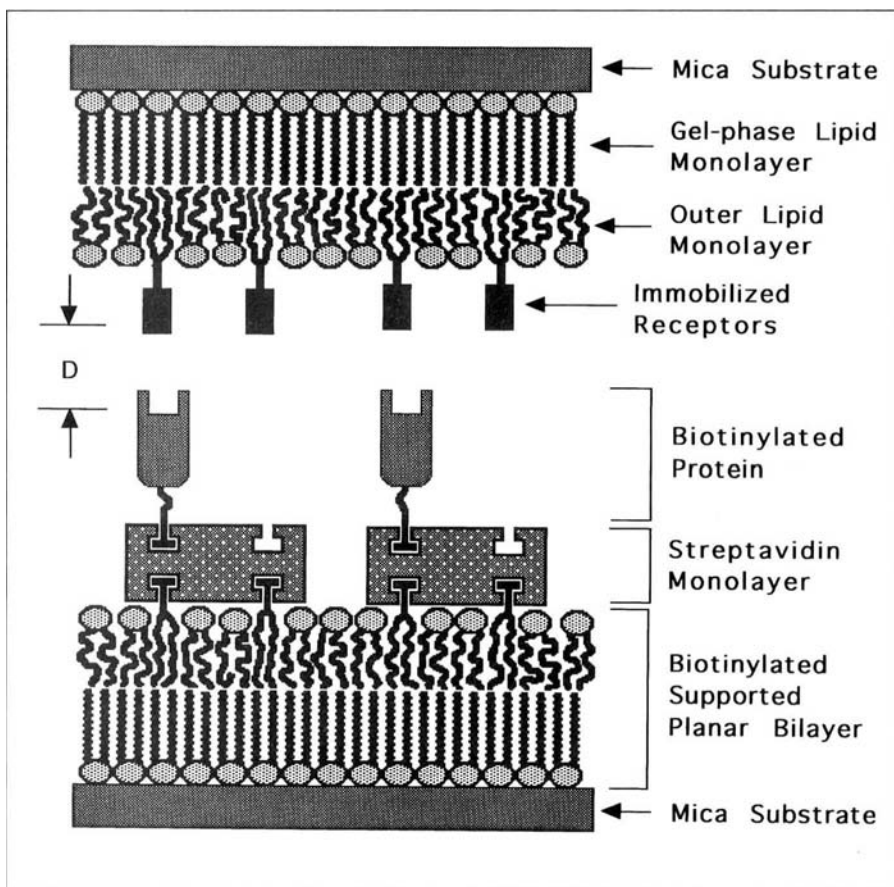


FIG 2. Schematic of the configuration of the bilayer-supported protein and receptor monolayers and their proposed relative orientations during force measurements. Supported bilayer membranes are prepared by Langmuir-Blodgett deposition, which permits the precise control of bilayer composition and lipid headgroup densities. Singly biotinylated proteins are specifically adsorbed to oriented streptavidin monolayers, immobilized on biotinylated membranes, or proteins can be directly bound to receptor-presenting or chemically activated surfaces. The forces between the two surfaces are measured as the distance, D , between them is decreased.

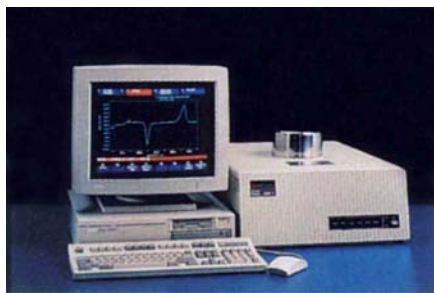
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A chemistry section

Force measurements can probe directly the molecular impact of proteins' electrostatic surface properties, specific bond strengths, membrane composition, and membrane fluidity on biological recognition events.

NEW **ion sensitive field effect transistor (ISFET) technology** is now offered by Beckman Instruments to provide an improved method for measuring pH (*Reader Service No. 101*). The ISFET pH system includes two pH meters and two probes. The Φ Smart ISFET microprobe is designed for molecular biology applications where measuring small volumes is essential. The tip of the microprobe measures 3.2 mm in diameter and easily fits into thin tubes and 96-well microtitre plates. The ISFET probes are said to require neither wet storage nor routine maintenance. Two meters are available in the Φ 100 ISFET series: the Φ 100 provides temperature-compensated pH (ATC) and mV results (absolute and relative), 1- and 2-point calibration, a recorder output, automatic buffer recognition and AutoRead for locking onto stable readings. The Φ 110 provides higher resolution, 3-point calibration with nonlinear curve fitting, storage and recall of results and an RS-232 interface for sending data to a computer or printer.

Perkin-Elmer has introduced a new **dynamic differential scanning calorimetry (DDSC) accessory** for the company's power-compensated DSC 7 differential, scanning calorimeter, which is designed to extend the characterization capabilities of the DSC technique (*Reader Service No. 102*). The new DDSC approach utilizes traditional multistep methods for data collection, making it easy to learn and use. The combination of traditional methods with the company's power-compensated

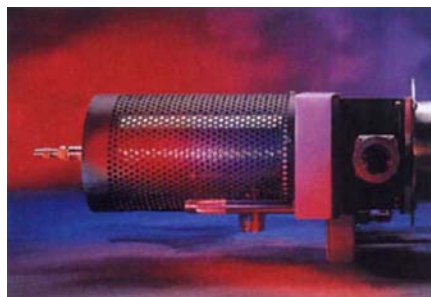


Perkin-Elmer's DSC 7 calorimeter.

sated DSC should provide improved temperature control during DDSC experiments. All data are quantitative and expressed in specific heat units. From the dynamic component of the sample response, storage and loss-specific heat curves, as well as tangent delta curves are obtained — both of which may be useful in further characterizing a viscoelastic materials in the glass transition region or during melting or crystallization. The data can also be helpful in separating different events that occur simultaneously in

a material, or it can provide complementary information to dynamic mechanical measurements, further identifying storage and loss of energy.

Hewlett Packard has introduced **atmospheric-pressure chemical-ionization liquid chromatography/mass spectroscopy** for its HP MS Engine system (*Reader Service No. 103*). The APCI technique is a soft ionization process applicable to the analysis of small, polar and nonpolar labile compounds. The technique can be applied to metabolite confir-



HP MS engine from Hewlett Packard.

mation and to the analysis of many pharmaceutical compounds and environmental contaminants. Using a time-programmable collision-induced dissociation (CID) feature, laboratories can optimize fragmentation of each eluting compound for maximum structural information. The manufacturer states that the sensitivity can be enhanced by up to ten times the nonpolarized value. The system utilizes the company's Windows-based LC/MS software and automates routine instrument-control and data-interpretation tasks and reduces the time required to learn the system. LC/MS software allows integrated LC and MS control for coordinated operation.

The CM200 FEG **transmission electron microscope** from Philips Electron Optics is said to be ideal for materials research targeted at structures on the atomic scale, such as interfaces, crystal structures and defects (*Reader Service No. 104*). It enables many types of information to be gathered at scales of 1 nm or smaller. The company has just produced a brochure outlining the key features of this innovative instrument. The CM200 FEG's combination of ultra-high resolution in TEM imaging, diffraction, microanalysis

and scanning, are all described in the brochure, supported by high-quality photographs and illustrations. The brochure also describes the microscope's CompuStage goniometer, twin-type objective lens and Schottky field emitter.

Software

Synopsis has developed Accord, a **chemical spreadsheet package** for Microsoft Excel (*Reader Service No. 105*). The program is designed to transform Excel into a spreadsheet that 'understands' chemistry — using the language of structural diagrams. This may prove to be an ideal tool for exploring chemical trends and QSAR studies. Chemical properties, such as molecular weight, can be calculated by linking an Accord function to a spreadsheet cell containing a chemical object. Producing an R-group table or filtering a chemical dataset by substructure is now handled through this Excel program. It can also provide 'cut-and-paste' compatibility with the major chemical drawing packages on the market, including ChemDraw, ISIS/Draw and ChemWindow.

For software developers, Molecular Simulations has introduced a kit called C² SDK, which allows programmers to **develop chemical research software** within a modelling and data interchange environment (*Reader Service No. 106*). Research groups can operate all their software in a single integrated user interface — the advantage being that individuals do not have to learn different pro-



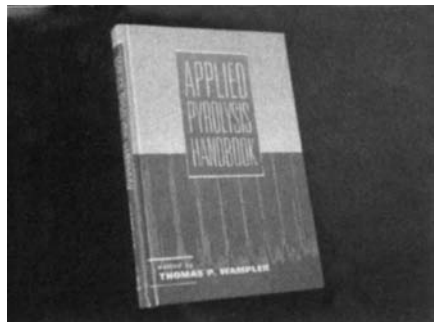
Molecular Simulations research software.

ocols when using different programs. This program is designed to allow researchers to integrate their own methodologies with commercial-quality modelling and simulation software. This program may prove to increase the speed and quality at which new software solutions are implemented. The 'rule book' feature uses a layer of interchangeable

modules for performing tasks common to many different kinds of research software.

Pyrolysis

The **applied pyrolysis handbook** from CDS Analytical contains the latest methods for analysing forensic evidence, as well as synthetic polymers, paints, fibres, natural substances and other materials (*Reader Service*



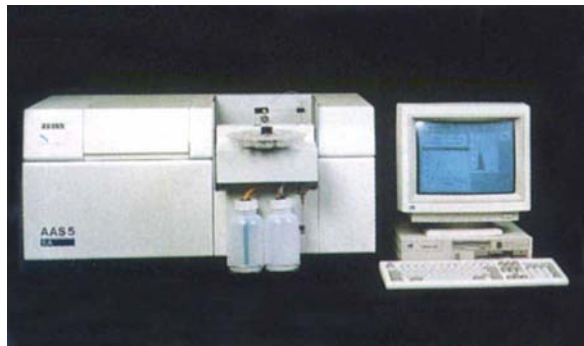
CDS applied pyrolysis handbook.

No. 107). Designed to be a daily-use reference, the handbook contains 376 pages of pyrolysis techniques, complete with illustrations and pyrograms. The text also compares new results obtained using the methods presented with previously published results.

A new eight-page colour brochure is available from Horizon Instruments describing how the Rapyd-400 **pyrolysis mass spectrometer system** can be used to discriminate between similar samples, to detect adulteration or counterfeiting and to quantify production processes (*Reader Service No. 108*). The brochure describes how the instrument can be used for microbial discrimination, comparison of polymers, adulteration of foods, country of origin studies and forensic examinations. The brochure also describes how the instrument works, how samples are prepared and how the acquired data are analysed using multivariate statistics and artificial neural-networking techniques.

Spectrometry

The AAS 5FL from Carl Zeiss is designed to be an efficient **trace detector** that uses automatic sequential flame technology for ultra-precise measurements (*Reader Service No. 109*). This spectrometer features high-aperture optics, a chopped-light technique with



Automatic sequential flame technology from Carl Zeiss.

high zero stability and automatic background compensation. Hollow-cathode and EDL lamps can be accommodated in the motorized six-place lamp changer, and a pre-heating circuit ensures readiness for operation after each element change. The instrument runs on software that is designed for complete control of the instrument and accessories, as well as the production of all necessary records.

Varian offers a comprehensive brochure describing the Cary 1/3 and Cary 4/5 **spectrophotometers and accessories** (*Reader Service No. 110*). The brochure highlights the important information needed to understand the capabilities of Cary instruments for performing solid sample measurements. It includes specifications for Varian's extensive array of unique solid sample handling accessories, such as diffuse reflectance, absolute specular reflectance and variable angle specular reflectance accessories. Each is described in detail and depicted in photographs or drawings.



Varian's brochure.

Shimadzu Scientific Instruments has introduced two new configurations of the GC/MS QP-5000, which have been designed to meet the requirements for the US Environmental Protection Agency's (**EPA**) **volatile and semivolatile methods** (*Reader Service No. 111*). The volatiles system incorporates the OI 4560 purge and trap concentrator coupled with either the QP-5000 standard (501 sec⁻¹) or high vacuum (1,511 sec⁻¹) system. The semivolatiles system is based on the QP-5000 standard vacuum system with the AOC-17 liquid injector. Both systems provide exceptional sensitivity, simple up front access to the source for maintenance and the pump-down speed and cleanliness of a turbomolecular pump. Additionally, these systems include specialized EPA method functionality in the software — a switch that invokes specific functions to comply with EPA Contract Laboratory Program methods. These include special dialogue boxes for judging tune criteria, sample login, automated sample schedule batches, such as tune criteria checks, continuing calibration tables and specialized report outputs.

Miscellaneous

The Q-SYS/1 has been developed by Clyde Computing to offer smaller organiza-

tions an affordable **LIMS system**, enabling them to meet the increasing demands for accurate analysis, accordance with specifications and consistency (*Reader Service No. 112*). Like the Q-SYS/2, this system automates the quality assurance of data, ensuring that information is accurately recorded, allowing results to be rapidly entered, accessed and reported by operators with little computer experience. The difference between the two systems lies in the more limited amount of data that can be stored. The Q-SYS/1 is suited to smaller operations and has the capacity for up to 100 tests to be defined. This also offers a solution to those larger groups who wish to try a LIMS system on just part of their operation, the system can be easily upgraded to Q-SYS/2 should the user wish to expand the system. Results are accepted directly from analytical instruments or simply entered manually as they become available.

Hi-Tech Scientific has been developed a new flow cell, which allows the versatile SFA-20 **stopped-flow, rapid-kinetics accessory** to be used in conjunction with the company's F-4500 rapid scan spectrofluorimeter (*Reader Service No. 113*). This new cell is designed to combine the precision mixing and stopping capabilities of the SFA-20 series with the fast data sampling rates of the F-4500, allowing fast solution kinetics to be studied. This new combination is said to be ideally suited for use with small quantities of novel or expensive materials, as a thermostat is used with small volumes solutions and



Stopped-flow rapid kinetics from Hi-Tech.

rapid scanning facilities allow low signal levels to be integrated, bringing higher sensitivity. Reactant solutions are mixed by a high-efficiency mixer just as they pass into the new flow cell. A steady-state equilibrium is established in the flow cell as the solutions pass through so that the resultant mixture only spends a few milliseconds as it passes through the spectrofluorimeter light beam. The system is stated to have an empirical deadtime of 15 milliseconds, allowing reaction rates of up to 0.01 s to be monitored. It is available in both two-syringe (250 µl to 5 ml) and multimixing versions.

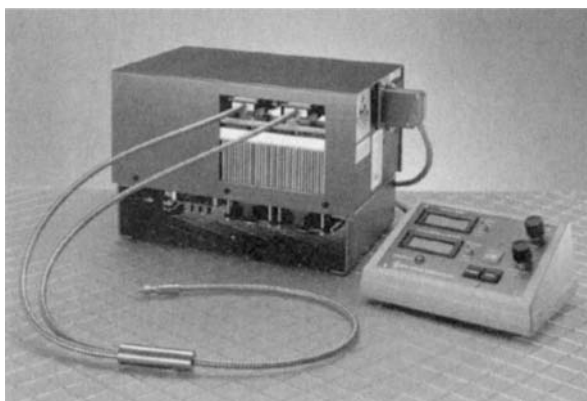
A new **automatic image analysis system** has been announced by Synoptics for use in routine materials and metallurgical applications (*Reader Service No. 114*). The Prosys system is designed to provide fast, cost-effective measurements of phases, inclusions, porosity and dimensions, such as layer thickness. The system is a self-contained, mouse-driven unit, which can be connected to an optical microscope or a macro-viewer to accommodate a wide variety of samples. Images of the samples to be measured are



Image analysis for materials applications.

displayed alongside easy-to-use control menus on a single, high-performance colour monitor. Automatic measurements are organized by colour-coding the features to be measured using automatic or manual grey-level discrimination. Manual critical dimension measurements can be made using pair-controlled callipers overlaid on the image of the sample.

Opto Power has introduced the OPC-B030-FCPS, an integrated **30-watt, fibre-coupled, high-power diode laser module**, which has been designed for direct thermal applications (*Reader Service No. 115*). This OEM unit has a nominal wavelength of 830 nm and comes equipped with a control box that enables the user to adjust the laser's drive current. An analog interface permits external control. For optimal performance, the operating temperature is preset at the factory. Engineered to deliver near-infrared CW laser energy via a 1.5-mm optical fibre bundle, this model can be equipped with an optional optical converter that reduces the diameter of the beam and allows the output power from the fibre



Opto Power's OPC-B030-FCPS fibre-coupled diode laser.

bundle to be injected into multimode fibres as small as 400 μm . A visible diode laser that emits an aiming beam collinear to the infrared output is also available.

The **Mystaire acid digestion scrubbers** from Misonix are available in microwave or hot plate configurations (*Reader Service No. 116*). The can be used for any acid, as well as many other fumes, vapours and odours. Acid fumes in the digestion hood are drawn into a two-stage scrubber where the vapours are cooled, absorbed and neutralized by the scrubbing solution with in the WaterWeb packing. The acid digestion scrubbers virtually eliminate acid emissions in the atmosphere and prevent the costly corrosion of hood, lab equipment and building ventilation systems.

The Spe-ed chiller, a new option for the Spe-ed SFE from Applied Separations, allows the operator to **use an entire cylinder of liquid CO_2** , resulting in significant savings (*Reader Service No. 117*). With this system no helium head pressure is required, resulting in an additional savings for helium. The company maintains that CO_2 with a helium head pressure is 15–30 per cent less dense than CO_2 alone — higher CO_2 density is said to lead to higher solubility resulting in shorter extraction times, less CO_2 consumption, and improved recovery of polar extractions. Work can be done in the subcritical liquid CO_2 range at a nominal 5,800 kPa to the high-pressure, supercritical CO_2 range up to 70,000 kPa. Variable micrometering valves give the user total control and flexibility over flow rate and temperatures on the system.

Fisher Scientific announces the release of their 1995–96 **chemical catalogue** (*Reader Service No. 118*). This edition includes over 1,300 new catalogue numbers representing more than 500 new chemical listings. Some of the new products are additions to existing lines, such as GC Resolv solvents and Fisher-Biotech chemicals. New product lines are also featured, including ScintiSafe liquid scintillation cocktails and several fine organic research chemicals from the company's chemical supplier Arcos Organics.

A new catalogue available from Romil lists the company's range of **high purity solvents, acids and reagents** for use in modern analytical techniques (*Reader Service No. 119*). The catalogue is divided into sections covering solvent, anhydrous solvents, acids and ion-pair reagents. Both organic and inorganic trace analysis are provided for by such techniques as HPLC, GC and UV/IR spectroscopy, as well as atomic absorption/emission spec-

troscopy. Advice on stability and storage is included, together with regulatory information and a number of useful reference charts for UV and IR spectroscopy.

J.T. Baker has introduced a comprehensive **chemical care programme** developed to increase awareness and understanding of chemical management issues within an organization (*Reader Service No. 120*). Topics addressed include: spill response, waste management, regulatory awareness and compliance, use and selection for safety equipment, proper chemical handling and chemical safety. □

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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Time to relax research use of patents

Simon Cohen

The conditions under which research is exempt from European patent law are ambiguous, and current judicial interpretation is too restrictive. A more liberal approach would be a welcome boost to small research-based companies.

SHOULD scientists be allowed to carry out research using a patented process without either a licence or the permission of the patent-holder? This question is being increasingly raised in both academic and industrial circles, not merely because companies are keen to receive royalties on their technological inventions, but also because the research for which these inventions are used may itself have commercial value. The common interpretation is that research is exempt from patent restrictions; but the reality is more complicated.

One area in which this issue arises is the use of a patented drug in clinical trials designed to test a treatment not covered by the original patent. The borderline between experimental and commercial use of patented subject matter has also come under scrutiny in the dispute between Promega and Hoffmann-La Roche over the use in academic laboratories of the thermostable enzyme *Taq* polymerase (see *Nature* 375, 348; 1995).

Another recent case that focused on the so-called 'research exemption' was the unsuccessful bid in the UK High Court by Murex Diagnostics Limited and Organon Teknika Limited to challenge the scope of a patent monopoly awarded to the US biotechnology company Chiron on making and selling test kits for hepatitis-C virus (see *Nature* 375, 348; 1995).

Murex had been developing an assay for serotyping the hepatitis-C virus when it was successfully sued by Chiron for patent infringement. Murex wanted to continue research and development on this particular assay, even though its commercial use would infringe Chiron's patent. Ambiguity in the scope of the statutory research exemption meant that, to avoid a further legal challenge, Murex had to reach an agreement with Chiron while awaiting the hearing of its appeal on the first charge.

Under the terms of UK and European patent law, work carried out for 'experimental purposes' on patented subject matter does not constitute an infringement of the patent concerned. But what does experimental purposes mean? It obviously covers fundamental research, including efforts to modify or improve the invention with no commercial goal in mind. But does it include research carried out to demonstrate to a potential customer that a product works, or clinical trials intended to

demonstrate to regulators that a product is safe and effective?

Such questions are often relevant in patent disputes where both sides have been carrying out research in parallel in the same field. Murex and Organon, if their challenge to Chiron's patent is unsuccessful, will be prevented from continuing to market their own hepatitis-C screening products. But they will also be prevented from developing their product without a licence from Chiron, unless such work is purely experimental.

Despite a prohibition on immediate sales, such companies may wish to continue to develop products — including their use in clinical trials — in order either to be ready to market such products as soon as the relevant patent expires (or is invalidated), or to apply for compulsory licences.

Some of these questions were considered by the UK Court of Appeal in a suit brought by Monsanto Co. against Stauffer Chemical Co. over the herbicide Touchdown. Before a patent challenge from Monsanto, Stauffer had carried out field trials with the herbicide and obtained limited safety clearances. Even though Monsanto's challenge was successful, Stauffer still wanted to carry out more trials to obtain further clearances.

The Court of Appeal ruled that trials carried out either to discover an unknown effect, or to test a hypothesis, can be regarded as experiments. But it added that trials carried out to demonstrate to a third party that a product works, or to collect information to satisfy either a customer or regulatory body, were not acts done for "experimental purposes".

The current law is therefore clear. But one equally clear result is that when a party loses an action for patent infringement, it may have to abandon the research that was the subject of the action, at least until expiry of the patent.

The potential problem for medical research was highlighted in the report *Intellectual Property & the Academic Community*, published earlier this year by the National Academies Policy Advisory Group (NAPAG) (see *Nature* 374, 398; 1995). If a patented substance is being studied for new therapeutic applications, it will at some stage have to be tested on patients — an activity that would constitute patent infringement.

In the United States, the research exemption is restricted to 'experimental or other nonprofit purposes'. Although the

wording is similar to that of European patent legislation, it has been interpreted by US courts as allowing clinical trials aimed at meeting regulatory requirements for new drugs.

US experience suggests that the scope of what is understood to be 'experimental purposes' in Europe should be widened and clarified. Such a move would have the additional advantage of bringing European and US legislation in line on the issue. This would not need a legal amendment, as the law could be clarified merely by a clear judicial ruling.

Ideally, this clarification would specify that activities such as validation tests should not be considered to infringe a patent. Only real commercial use, involving a financial transaction, should be prohibited. This would enable companies who are successfully sued for infringing a patent — or who fail in an attempt to challenge a patent held by another company — to continue to carry out research in an area where they may already be well advanced.

Research of this type could result in a useful modification of the patented invention, which the original patent owner may wish to license or may agree can be produced and sold under licence. Alternatively, the research could lead to a product or process which is sufficiently distinguishable from the original patent that it requires no cooperation from the patent-holder.

Scientists must be given incentives to develop innovative ideas and products, and this means allowing them the monopoly period provided by patents. But at the same time, competitors should be allowed to experiment with such products during the period of the monopoly.

To forbid any unlicensed activity apart from fundamental research during the lifetime of a patent wastes the investment that others have made in the area. Even worse, it risks paralysing associated research. The current interpretation of the research exemption in Europe places a particular burden on research-based companies that may have several promising products in the pipeline but few (if any) generating revenue. A more liberal interpretation would limit the 'winner takes all' effect, and would thus act as a welcome boost to such companies as they strive to get off the ground. □

The author is a solicitor at Taylor Joynson Garrett, 50 Victoria Embankment, London EC4Y 0DX, UK.

This is one of an occasional series of articles on intellectual property and the law.